UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

March 1, 2005

BIOMEDICAL GRADUATE PROGRAMS

Ph.D. Degrees

Interdisciplinary
-Emerging Infectious Diseases
-Molecular & Cell Biology
-Neuroscience

Departmental
-Clinical Psychology
-Environmental Health Sciences
-Medical Psychology
-Medical Zoology
-Pathology

Doctor of Public Health (Dr.P.H.)

Physician Scientist (MD/Ph.D.)

Master of Science Degrees

-Molecular & Cell Biology
-Public Health

Masters Degrees

-Comparative Medicine
-Military Medical History
-Public Health
-Tropical Medicine & Hygiene

Graduate Education Office
Dr. Eleanor Metcalf, Acting Associate Dean
Janet Anastasi, Program Coordinator
Heather DeLloyd, Educational Assistant

Web Site
www.usuhs.mil/geo/gradpgm_index.html

E-mail Address
graduateprogram@usuhs.mil

Phone Numbers
Commercial: 301-295-9474
Toll Free: 800-772-1747
DSN: 295-9474
FAX: 301-295-6772

APPROVAL SHEET

Title of Dissertation: "Hypoxia-Inducible Factor Prolyl Hydroxylases are Oxygen Sensors in the Brain"

Name of Candidate: Clifton Dalgard
Doctor of Philosophy Degree
11 March 2005

Dissertation and Abstract Approved:

Brian Cox, Ph.D. — 3/29/05 — Date
Department of Pharmacology
Committee Chairperson

Ajay Verma, M.D., Ph.D. — 3/29/05 — Date
Department of Neurology
Committee Member

Aryan Namboodiri, Ph.D. — 3/22/05 — Date
Department of Anatomy, Physiology & Genetics
Committee Member

David Grahame, Ph.D. — 3/28/05 — Date
Department of Biochemistry
Committee Member

Ignacio Provencio, Ph.D. — 3/11/05 — Date
University of Virginia
Committee Member

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAR 2005		**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Hypoxia-Inducible Factor Prolyl Hydroxylases are Oxygen Sensors in the Brain	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences (USUHS),4301 Jones Bridge Road,Bethesda,MD,20814-4799**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**125**	

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

11 march 2005

MEMORANDUM FOR DR. BRIAN COX, DEPARTMENT OF PHARMACOLOGY, CHAIRMAN FOR CLIFTON DALGARD'S THESIS COMMITTEE.

SUBJECT: Clifton Dalgard's Contribution to Thesis-related Publications

Clifton Dalgard's Graduate Dissertation includes one published manuscript, one submitted manuscript and one manuscript in preparation. Since two of these manuscripts have co-first authors, this memorandum serves to define Clifton's contribution to these.

In the published manuscript entitled "ENDOGENOUS 2-OXOACIDS REGULATE EXPRESSION OF OXYGEN SENSORS" Clifton performed all of the work and received some assistance with the HIF-1α western blots and the HRE-luciferase experiments. The main reason that Dr. Huasheng Lu was listed as a co-first author is that he initially produced the data identifying oxaloacetate and pyruvate as endogenous regulators of HIF-1α.

In the submitted manuscript entitled "REVERSIBLE INACTIVATION OF HIF PROLYL HYDROXYLASES BY CANCER CELL METABOLISM CONTROLS BASAL HIF-1" Clifton performed all of the gene expression analyses. This included designing all RT-PCR primers, verifying the products, performing the cell treatments and extractions, and quantifying mRNA expression. Clifton also devised the assay for binding between HIF-1α and pVHL, as well as the assay for binding between 2-oxoglutarate and the HIF prolyl hydroxylases. This includes in vitro translations of all ^{35}S labeled proteins, generation of the affinity gels, cell treatments and cell extractions.

The third manuscript, which is still in preparation, will feature Clifton as the sole first author.

Ajay Verma

Ajay Verma, M.D., Ph.D.
Associate Professor of Neurology
Thesis Advisor for Clifton Dalgard

COPYRIGHT STATEMENT

The author herby certifies that the use of any copyrighted material in the thesis manuscript entitled:

"Hypoxia-Inducible Factor Hydroxylases Are Oxygen Sensors in the Brain"

is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Clifton Lee Dalgard
Neuroscience Program
Uniformed Services University of the Health Sciences

ABSTRACT

Title of Dissertation:	"Hypoxia-Inducible Factor Hydroxylases Are Oxygen Sensors in the Brain"
Name:	Clifton Lee Dalgard, Ph.D. 2005
Dissertation directed by:	Ajay Verma, M.D., Ph.D. Associate Professor Uniformed Services University of the Health Sciences

In mammalian cells, molecular oxygen is a requirement for normal growth, metabolism, and survival. Tissues in which oxygen demand surpasses oxygen supply become hypoxic and cannot maintain normal function. Thus, the ability to sense oxygen levels is necessary for an organism to survive and thrive in changing environmental and physiological conditions. HIF-1 is a transcription factor complex that is essential and central to several cellular and systemic adaptations to hypoxia. For example, vascular endothelial growth factor and erythropoietin are HIF-1 target genes that are important in angiogenesis and erythropoiesis, respectively. HIF-1 consists of two subunits, alpha and beta, and control of HIF-1 function is accomplished through the hydroxylation of proline residues and an asparagine residue on the α-subunit of HIF-1. Under normoxic conditions, hydroxylated HIF-1α is constantly and rapidly degraded, thus HIF-1 is inactivated. Additionally, undegraded HIF-1α is hydroxylated at an asparagine residue in the c-terminal region, which prevents it from binding to the co-transcriptional activator p300. The post-translational modifications of HIF-1α are performed by four oxygen-dependent enzymes, the three HIF-1α prolyl hydroxylases (HPH-1, HPH-2, and HPH-3) and the asparaginyl hydroxylase FIH-1 (Factor Inhibiting HIF). Since these enzymes

modify HIF-1α in an oxygen-dependent manner, they have been suggested to function as oxygen sensors *in vivo*. No studies of these oxygen sensors have been conducted in the mammalian brain or brain derived cells. This dissertation describes biochemistry, cellular and molecular biology, and whole animal physiology of these oxygen sensors. Using human glioma cell lines, we demonstrate that HPHs are themselves induced by hypoxia, thus suggesting the presence of a negative feedback system to modulate hypoxic gene expression. For the three HPHs, we found differential distribution of expression between different brain cell types and different brain regions. The same HPH homologues that are regulated in permanent cell lines are regulated in brain cells in culture and *in vivo*. We found that different brain regions induce HPH expression to different extents and hypoxic induction of the oxygen sensors was more prominent in young animals than in old and was manifested by increases in protein expression and enzymatic activity. We also found in addition to oxygen availability, the HIF hydroxylases are also regulated by certain glycolytic metabolites. We specifically identified pyruvate and oxaloacetate as the regulatory metabolites and demonstrated that their mode of action involves a reversible inactivation of HIF hydroxylation. Pyruvate and oxaloacetate induced HIF-1 in cells and also resulted in upregulation of HPH-1 and HPH-2. These results suggest HIF prolyl hydroxylases are sensor of oxygen tensions as well as glycolytic metabolite accumulation. Moreover, both of these stimuli increase expression of these hydroxylases which may serve as a negative feedback system for these sensing mechanisms. Given that the brain is highly sensitive to low oxygen tensions, these studies may provide valuable insight to develop novel tools and therapies for oxygen-associated brain diseases like stroke, heart failure, and brain cancer.

HYPOXIA-INDUCIBLE FACTOR HYDROXYLASES ARE OXYGEN SENSORS IN THE BRAIN

by

Clifton Lee Dalgard

Dissertation submitted to the Faculty of the

Neuroscience Graduate Program of the

Uniformed Services University of the Health Sciences

in partial fulfillment of the requirements for the

degree of Doctor of Philosophy, 2005

DEDICATION

This dissertation is dedicated to the friendship and memory of Dr. Cinda Helke. She was the Neuroscience Program Director and the Associate Dean of Graduate Education who mentored and encouraged me during my studies at USUHS. Cinda's courage, strength, and continual dedication to education during the last year of her life provided me with an example of how to thrive under adversity and encouraged me to excel as a young professional. I miss her dearly and will remember her in all my accomplishments.

ACKNOWLEDGEMENTS

To my wife Elizabeth, you are the only reason I need to be hopeful for the future. I look forward to each and everyday in our lives…without a dissertation to complete.

To my sister Jackie, you led the way and I appreciate all the enthusiastic support and professional opinions along the way. The examples you made during your graduate education and dissertation defense were positive motivation for my education and helped shape my career goals.

To my mother Ejen, thank you for providing me with the foundation I needed to make it thus far. I would not have the ability without your lessons and nurturing love in my life.

To my mentor Ajay, your patience and wisdom are truly limitless. At times I wanted to look away from science, but you provided the reasons why science is a part of both of our lives. Every experiment I do from this time forward will have your training and expertise guiding me.

To the members of my dissertation committee, Dr. Brian Cox, Dr. David Grahame, Dr Iggy Provencio, and Dr. Aryan Namboodiri, thank you for your time and experise to improve my experience and work during these last 3 years. Your contributions were always welcomed and appreciated.

To the many friends I have met these last four years, I must acknowledge all the assistance, advise, and support you have provide over the years. Especially, I need to express my gratitude and deep appreciation to John and Katie Pesce and Ahmed Mohyeldin whose friendship, hospitality, and wisdom have supported and greatly entertained me over the last few years. You have consistently helped me keep perspective on what is important in life and shown me how to deal with hardship as well as festivity.

TABLE OF CONTENTS

Approval Sheet		i
Memorandum to the Thesis Committee		ii
Copyright Statement		iii
Abstract		iv
Title Page		vi
Dedication		vii
Acknowledgements		viii
Table of Contents		ix
Introduction		1-8
Summary of Paper 1		9-10
Paper 1 -	"Endogenous 2-oxoacids differentially regulate expression of oxygen sensors"	
Summary of Paper 2		11-13
Paper 2 -	"Reversible inactiviation of HIF prolyl hydroxylases by endogenous 2-oxoacids allows HIF-1 to serve as a metabolic sensor"	
Summary of Paper 3		14-15
Paper 3 -	"Differential regional expression of the HIF prolyl hydroxylase mammalian oxygen senors in the rat brain"	
Discussion		17-23
Bibliography		24-31

INTRODUCTION

Vertebrate cells possess adaptive responses to hypoxia.

Multicellular organisms have a dependency for oxygen, which is necessary in energy metabolism for continued growth, development, and survival. Thus, it is not surprising to find both systemic and cellular mechanisms to maintain appropriate oxygen homeostasis in vertebrate organisms. Short and long term cellular adaptations are seen in cells and in organisms after exposure to acute and chronic hypoxia, respectively (Bickler and Donohoe 2002). Short-term cellular adaptations have been demonstrated in oxygen-sensitive ion channels, where ion conductance is decreased during hypoxia (Fearon, Palmer et al. 1999; Fearon, Palmer et al. 2000; Fearon, Randall et al. 2000; Lewis, Hartness et al. 2001). Production of reactive oxygen species during hypoxia is also thought to yield short-term adaptations through signaling by the mitogen-activated protein kinase system (MAPK) (Haddad and Land 2000), which may lead to acute suppression of metabolism and thus, reduction of oxygen demand. Additionally, MAPK p42/42, which is associated with neuroprotective gene expression, is activated in a phospholipase C mediated manner during acute hypoxia (Donohoe, Fahlman et al. 2001). Perhaps the most important responses to hypoxia are found in mechanisms that lead to long-term transcriptional regulatory changes. These mechanisms include the increased gene expression of erythropoietin (Epo) (Ruscher, Freyer et al. 2002) and vascular endothelial growth factor (VEGF) (Wick, Wick et al. 2002) to increase oxygen supply by stimulation of red blood cell production and vascularization, respectively. Additionally, upregulation of genes for anaerobic glycolysis occurs (Semenza, Roth et al. 1994; Marrif

and Juurlink 1999; Ouiddir, Planes et al. 1999), most likely to decrease the oxygen demand by a cell during ATP energy production. These above mechanisms for hypoxic adaptation are mediated by a key transcription factor known as hypoxia-inducible factor (HIF-1), whose protein levels are increased under low oxygen (Wang, Jiang et al. 1995; Jiang, Semenza et al. 1996). The recent identification of four oxygen-dependent enzymes (HPH-1, HPH-2, HPH-3, and FIH-1) (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Mahon, Hirota et al. 2001; Lando, Peet et al. 2002) which modify the alpha subunit of HIF-1 and lead to the HIF-1 inactivity has now provided us with targets for pharmacological inhibition in efforts to stimulate hypoxic adaptation before cell death. The studies of these enzymes and oxygen sensing mechanisms were performed in mainly in renal clear cell (Krieg, Haas et al. 2000), HeLa (Wang and Semenza 1993), and Caenorhabditis elegans cell types (Epstein, Gleadle et al. 2001), and are poorly understood in neuronal and glial cells, thus, the research described in this thesis is significant, because it will provide knowledge about hypoxic adaptation mechanisms in the central nervous system, and may explain the differential susceptibility of brain regions to hypoxic injury. Additionally, the findings may offer insight into the development of neuroprotective therapeutic strategies through stimulation of these adaptation mechanisms in brain cells.

Hypoxic sensitivity varies among brain regions and cells.

Cerebral hypoxia has been established as an important element contributing to brain damage during stroke and several other afflictions leading to reduced oxygen supply to the brain (asphyxiation, cancer, cardiac arrest, etc.). Neurons are considered to be the most oxygen sensitive of all vertebrate cells. However, recent studies demonstrate

that some vertebrate neurons possess the ability to live without oxygen for long durations (Hochachka and Lutz 2001). Thus, neuronal response to oxygen deprivation can demonstrate differential sensitivity and injury. For example, one study demonstrated that after hypoxic/ischemic injury, neuronal loss is most prominent in the hippocampus, globus pallidus, cerebellum, and inferior olives (Dijkhuizen, Knollema et al. 1998). In the same study, neuronal injury was significantly less in the substania nigra and diencephalic thalamus. Even within a specific brain region, cells may display differential sensitivity to hypoxic insult. In the rat brain, it has been shown that cells in the CA1 and CA3 region of the hippocampus are more vulnerable to hypoxia than cells in the adjacent CA2 and CA4 hippocampal regions (Kawaguchi, Takizawa et al. 2002). The underlying reason for the differential sensitivity of these cells and others in the brain remains entirely unknown.

Hypoxia-inducible factor 1 regulates adaptation to hypoxia.

Oxygen homeostasis is a critical necessity for mammalian cells and the transcription factor complex hypoxia-inducible factor 1 (HIF-1) is a key regulator of the many adaptations available to maintain appropriate cellular oxygen levels (Czyzyk-Krzeska 1997; Wenger and Gassmann 1997; Semenza 1999). HIF-1 was identified during the study of erythropoietin gene induction in the kidney under hypoxia (Wang and Semenza 1993; Wang and Semenza 1993). It was found that HIF-1 was a factor that bound to DNA at the 3' end of the erthyropoietin gene only after hypoxia exposure(Wang and Semenza 1993). The cloning and subsequent analysis of HIF-1 revealed its composition of two subunits, a 120 kDa α subunit and a 91 kDa β subunit (Hirose, Morita et al. 1996). Analysis of the HIF-1α subunit provided interesting results as the protein

was subject to rapid turnover and degradation under normal oxygen levels, but significantly accumulated under hypoxia (Wang, Jiang et al. 1995; Jiang, Semenza et al. 1996). In contrast, the mRNA of HIF-1α, as well as the mRNA and protein levels of the β subunit did not alter significantly with changes in oxygen levels (Wenger, Kvietikova et al. 1997). Even though the molecular mechanism by which cells "sensed" oxygen levels and transformed the physiological signal to changes in HIF-1α protein levels remained unknown for a long time, this protein stability property of HIF-1α helped to establish the regulatory mechanism of HIF-1 target genes under hypoxia and helped identify numerous genes that possess a hypoxia response element (HRE) which is transactivated by HIF-1 (Bernaudin, Tang et al. 2002). Most of these proteins have a direct role in oxygen homeostasis, and upregulation of these genes in various experimental models (for example, hypoxic preconditioning) leads to increased survival under low oxygen.

Hypoxic preconditioning may be mediated via HIF-1.

Long before HIF-1 was identified, it was suggested that exposure to hypoxia would be able to induce endogenous adaptive mechanisms that can protect the brain from hypoxic-ischemic injury. This was found to be true as studies in the early 1990s demonstrated that exposure to acute hypoxia could confer neuroprotection from subsequent lethal ischemic episodes in both perinatal and adult rats (Gidday, Fitzgibbons et al. 1994). More specifically, 3 hour exposure to 8% O_2, 24 hours prior to a hypoxic/ischemic insult reduced infarction 34% as compared to vehicle-treated control animals. Proteins involved in the endogenous adaptive response to hypoxia were unknown at the time, however, after the identification of HIF-1, it was quickly discovered that the expression of HIF-1α, as well as several HIF-1α target genes (aldolase, glucose

transporter 1, lactate dehydrogenase, phosphofructokinase, and vascular endothelial growth factor), were increased after preconditioning with hypoxia and that this increase was correlated with a 96% brain protection compared with littermate control animals after hypoxic/ischemic insult (Bergeron, Gidday et al. 2000). Additionally, it was found that non-hypoxic mediated increases of HIF-1α, by injection of cobalt chloride, lead to similar increases in gene expression of HIF-1α target genes and a 76% brain protection compared with controls. Thus, HIF-1 was found to directly contribute to establishment of hypoxic/ischemic tolerance. Molecular targets which may increase HIF-1α levels in normoxic conditions are now of interest, as the known hypoxic-mimetics, cobalt chloride and desferrioxamine, display cytotoxicity (Tam, Leung-Toung et al. 2003) in tissue and are not recommended for use in humans.

Novel "oxygen sensors" regulate HIF-1.

Identification of the "oxygen sensors" responsible for changes in HIF-1α protein levels began with the finding that the degradation of HIF-1α occurred by the ubiquitin proteasome pathway as proteasomal inhibitors or mutations in the ubiquitination pathway resulted in elevated levels (Salceda and Caro 1997). Fusion protein studies of HIF-1α identified a region that contributed to the ubiquitin-mediated degradation and was termed the oxygen degradation domain (ODD) (Huang, Gu et al. 1998). Removal of the ODD domain generated a stable, non-oxygen inducible HIF-1α protein that constitutively activated HIF-1 target genes (Huang, Gu et al. 1998). Mutation analysis of the ODD domain finally identified two proline residues that promoted degradation and that hydroxylation of these two prolines was responsible for targeting of HIF-1α to ubiquitination by the E3 ligase protein, von Hippal Lindau (pVHL) (Epstein, Gleadle et

al. 2001; Jaakkola, Mole et al. 2001). This poly-ubiquitination after prolyl hydroxylation results in proteasome dependent degradation of the HIF-1α protein. Genetic analysis and molecular cloning of the gene responsible for the prolyl hydroxylation of HIF-1α occurred in Caenorhabditis elegans and identified the activity of the egl-9 gene, a prolyl hydroxylase (Epstein, Gleadle et al. 2001). Not surprisingly, C. elegans mutants for egl-9 were found to constitutively express HIF-1α. Database searching for homologs of egl-9 in humans identified three separate mammalian prolyl hydroxylases termed HPH 1, 2, and 3 (Bruick and McKnight 2001). Recent in vitro biochemical analysis showed that these enzymes utilize molecular oxygen and 2-oxoglutarate as cosubstrates and iron as a cofactor to catalyze the hydroxylation of proline-402 and proline-564 on HIF-1α (Bruick and McKnight 2001). Additionally, the enzymes were demonstrated to be inhibited by treatments of the hypoxia-mimetics cobalt chloride and desferrioxamine, which also induce HIF-1α in vivo. Most importantly, the role for the HPHs as an oxygen sensor was demonstrated by the reduction of HIF-1α modification under hypoxic conditions that induce HIF-1α in vivo (Pugh, O'Rourke et al. 1997).

HIF-1α also contains a carboxyl-terminal transactivation domain (CAD) which was found to be activated under hypoxic conditions (Pugh, O'Rourke et al. 1997). Functional analysis and yeast two-hybrid methods identified that under hypoxia, HIF-1α binds to CBP/p300, a transcriptional coactivator, which aids in transactivation of HIF-1 target genes (Kallio, Okamoto et al. 1998; Carrero, Okamoto et al. 2000; Kung, Wang et al. 2000). In experiments similar to those that led to the identification of the HPHs, it was found that an enzymatic hydroxylation activity modified HIF-1α to block p300 binding under normoxia (Lando, Peet et al. 2002; Sang, Fang et al. 2002). However, in

contrast to the modification of proline residues in ODD, it was found that asparagine was the amino acid residue modified in HIF-1α CAD (Lando, Peet et al. 2002). The asparagine hydroxylase was termed factor-inhibiting HIF (FIH-1) and demonstrated the same biochemical properties as the HPHs (Mahon, Hirota et al. 2001). FIH-1 required molecular oxygen and 2-oxoglurate as cosubstrates and could be inhibited by hypoxic conditions as well as hypoxia-mimetics. Thus, these four proteins mainly because of their actions on HIF-1α as well as their dependency on molecular oxygen as a substrate now represent the primary oxygen sensors in mammalian cells and are a focal regulatory point for hypoxic adaptive mechanisms that could be utilized to activate the neuroprotective effects of hypoxic preconditioning.

HIF-1α is induced by glucose metabolism.

In addition to its well-known activation by hypoxia, HIF-1 is also activated by glycolytic end products (Lu, Forbes et al. 2002). Our laboratory observed a high basal protein expression of HIF-1α in human gliomas which was found to be dependent upon glucose metabolism. This high basal level of HIF1α protein in gliomas was observed even when cultured in normoxic (21%O_2) conditions in minimal essential media (MEM). We had noted a positive correlation between fresh culture medium replacement and HIF-1α protein levels. By switching from culture medium to the relatively simple Krebs buffer, we were able to discover the dependency of HIF-1α levels on the metabolism of glucose to pyruvate. Further studies demonstrated that the glycolytic metabolites, pyruvate and oxaloacetate, are key regulators of HIF-1α protein levels and HIF-1 mediated gene expression in gliomas. However, the molecular targets on which pyruvate and oxaloacetate act to induce HIF-1α are entirely unknown. The identification of the

HIF hydroxylases / oxygen sensors provides a set of molecular targets to test for interaction with glycolytic metabolites.

Although much is now known about the biochemical properties of these "oxygen sensors", there are many questions to be answered. The anatomical and cellular distribution of these genes is not well known, especially in the central nervous system. Whether the individual HPH family members, which all have been shown to modify HIF-1α in vitro, can be selectively regulated by glycolytic metabolites remains a significant question. Additionally, the potential that these enzymes may have targets other than HIF-1α is unknown. Thus, many exciting questions remain in this area of research into cellular adaptation to hypoxia. In conclusion, review of the scientific literature identifies the significant progress that has been made in understanding the oxygen sensing signaling pathways, hypoxia adaptation, and potential therapeutic targets. In this thesis, we extend this progress through investigating the regulation (Paper 1), biochemistry (Paper 2) and expression (Paper 3) of the oxygen sensors in the area of the brain.

SUMMARY FOR PAPER 1

Rationale: In addition to molecular oxygen, HPH homologues require 2-oxoglutarate to catalyze hydroxylation of proline residues in HIF-1α. Endogenous 2-oxoacids that resemble 2-oxoglutarate may inhibit the activity of these enzymes, thus causing accumulation of HIF-1α independently of hypoxia. Activiation of HIF-1 by hypoxia or 2-oxoacids may in turn upregulate the expression of HPHs as a feedback mechanism.

Objectives: In this paper, we set out to (a) determine whether hypoxia could upregulate HPH homologues at the mRNA, protein, and activity level, (b) determine whether the 2-oxoacids, pyruvate and oxaloacetate, could cause accumulation of HIF-1α and subsequently induce expression of HPH homologues, and (c) determine whether an induction of HPH expression and enzymatic activity could negatively regulate HIF-1α protein expression.

Methods: These studies were performed in human glioma cell lines. We exposed glioma cells to either hypoxia, known hypoxia mimetics, or 2-oxoacids. HIF induction was examined by western blot analysis of nuclear extracts, as well as immunohistochemisty. HIF-1 mediated gene expression was examined by analysis of HIF activiation using a lucerifase reporter construct containing a HIF responsive element (HRE) as well as quantitative PCR analysis of HIF-1 regulated gene expression. HPH expression was analyzed by quantative PCR and western blotting. Changes in HPH activity were determined by an *in vitro* VHL-pulldown hydroxylation assay and by examining HIF-1α protein decay kinetics after reoxygenation.

Findings: HPH-1 and HPH-2 mRNA and protein as well as total cellular hydroxylation activity is induced by hypoxia in human glioma cells. HIF-1α accumulation as well as HPH-1 and HPH-2 induction occurs with hypoxia, hypoxia-mimetics, and the 2-oxoacids, pyruvate and oxaloacetate. Induction of HPH hydroxylation activity leads to an increase in HIF-1α protein decay following reoxygenation.

Significance: This paper demonstrated that glucose derived 2-oxoacids can regulate HIF-1 independently of hypoxia. This finding may help explain the presence of HIF-1α protein in normoxic cancer cells. We also showed both hypoxia and the 2-oxoacids were capable of inducing HPHs as a feedback mechanism for regulating HIF-1α protein levels. The reduction of HIF-1α protein levels in specific tissues during prolonged hypoxia may be explained by this negative feedback system.

Endogenous 2-oxoacids differentially regulate expression of oxygen sensors

Clifton Lee DALGARD[1], Huasheng LU[1], Ahmed MOHYELDIN and Ajay VERMA[2]

Department of Neurology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, U.S.A.

Adaptations to change in oxygen availability are crucial for survival of multi-cellular organisms and are also implicated in several disease states. Such adaptations rely upon gene expression regulated by the heterodimeric transcription factors HIFs (hypoxia-inducible factors). Enzymes that link changes in oxygen tensions with the stability and transcriptional activity of HIFs are considered as oxygen sensors. These enzymes are oxygen-, iron- and 2-oxoglutarate-dependent dioxygenases that hydroxylate key proline and asparagine residues in HIFα subunits. The constitutive inhibitory action of these enzymes on HIFs is relieved by hypoxia and by agents that displace iron or 2-oxoglutarate. Two of the enzymes, HPH (HIF prolyl hydroxylase)-1 and HPH-2, are known to be inducible by hypoxia in a HIF-dependent manner. This suggests the existence of a novel feedback loop for adjusting hypoxia-regulated gene expression. We have recently shown that HIF-1α stability, HIF-1 nuclear translocation and HIF-mediated gene expression in human glioma cell lines can be stimulated by pyruvate independently of hypoxia. In the present study we show that the endogenous 2-oxoacid oxaloacetate can also activate HIF-mediated gene expression. Pyruvate and oxaloacetate treatment of cells also up-regulates HPH-1 and HPH-2, but not HPH-3 or the HIF asparaginyl hydroxylase FIH-1 (factor inhibiting HIF). Regulation of HIF-1 and the expression of HPH homologue genes can thus be influenced by specific glycolytic and tricarboxylic acid cycle metabolites. These findings may underlie important interactions between oxygen homoeostasis, glycolysis, the tricarboxylic acid cycle and gluconeogenesis.

Key words: hypoxia, hypoxia-inducible factor (HIF), oxaloacetate, oxygen sensor, prolyl hydroxylase, pyruvate.

INTRODUCTION

Hypoxia induces adaptive responses in many organisms via the hypoxia-inducible factor family of transcriptional factors (HIF-1, HIF-2 and HIF-3), which up-regulate genes involved in glycolytic energy metabolism, cell growth and survival, angiogenesis and erythropoiesis [1]. Members of the HIF family are heterodimers comprising HIFα and HIFβ subunits [2], both of which are constitutively expressed in mammalian cells. Regulation of the HIF-1 complex is mainly dependent upon degradation of the HIF-1α subunit. Under normoxic conditions, HIF-1α is targeted for ubiquitinylation and proteasomal degradation via the E3 ligase component pVHL (von Hippel–Lindau protein), a tumour suppressor [3,4]. This process requires the binding of pVHL to an ODD (oxygen-dependent degradation domain) on the HIF-1α protein [5]. Enzymic incorporation of oxygen into the HIF-1α ODD is required to generate the binding site for pVHL [6], thus subjecting this process to regulation by oxygen availability.

Three distinct HIF prolyl hydroxylases (HPH-1, HPH-2 and HPH-3) have been identified, which regulate the binding of pVHL to HIF-1α by using molecular oxygen to hydroxylate proline residues 402 and 564 on the HIF-1α ODD [7–9]. HIF-1α is also hydroxylated at Asn^{803} by another hydroxylase known as FIH-1 (factor inhibiting HIF) [10–12]. Hydroxylation of Asn^{803} prevents HIF from interacting with the transcriptional co-activator p300 and therefore blocks HIF-mediated transcriptional activation [10–12]. By virtue of their ability to regulate HIF, the HPH homologues and FIH-1 are together referred to as oxygen sensors. These enzymes require oxygen, iron and 2-oxoglutarate for their activity. This explains why the hypoxic stimulation of HIF-1α protein stabilization and HIF-mediated gene transcription is mimicked by the iron chelator DFO (desferrioxamine), the iron-displacing metal cobalt or synthetic 2-oxoglutarate antagonists, such as DMOG (dimethyloxalylglycine) [7–12]. In addition to these regulatory factors, forced over-expression of each HPH homologue and FIH-1 has been shown to blunt HIF activation by hypoxia in cultured cells [13]. This suggests that the ability of cells to make transcriptional responses to hypoxia may be modified via altered expression of the HIF hydroxylases. Differential expression of the three HPH homologues and FIH-1 amongst various mammalian organs supports this notion [14,15].

The *hph-1* and *hph-2* genes have been shown to be inducible by hypoxia [7,13,16,17]. This up-regulation appears to underlie previous observations that the rate of HIF-1α decay following hypoxic induction depends upon the duration of the hypoxic period, with longer hypoxic periods promoting a more rapid degradation of HIF-1 upon re-oxygenation [18]. HIF activation is itself required for the hypoxic induction of HPH homologues, since this induction is blocked by HIF-1α small interfering RNA [19] and is absent in *hif1*$\alpha^{-/-}$ cells [17]. HIF-dependent HPH induction thus appears to represent a novel feedback mechanism for blunting HIF-induced gene expression during and after prolonged periods of hypoxia.

Although hypoxia is the ubiquitous inducer of HIF-1α in all cells tested, other stimuli, such as cytokines, growth factors, reactive oxygen species and nitric oxide, can also activate HIF under normoxic conditions (for review, see [20]). It is not known whether these stimuli also regulate expression of HPH homologues or of FIH-1. We have recently identified the glucose metabolite pyruvate (2-oxopropanoate) as a normoxic regulator of HIF [21]. Pyruvate was shown to increase HIF-1α levels, promote HIF-1 DNA binding and increase mRNA levels of several genes known to be regulated by HIF in human gliomas and other cell types, even in the presence of oxygen [21]. In the present study we

Abbreviations used: CA9, carbonic anhydrase IX; DFO, desferrioxamine; DMOG, dimethyloxalylglycine; HIF, hypoxia-inducible factor; FIH-1, factor inhibiting HIF; HPH, HIF prolyl hydroxylase; HRE, hypoxia-response element; IAA, iodoacetate; ODD, oxygen-dependent degradation domain; pVHL, von Hippel–Lindau protein, TCA cycle, tricarboxylic acid cycle; RT, reverse transcriptase.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail averma@usuhs.mil).

demonstrate direct activation of a HIF-regulated promoter by the glycolytic metabolite pyruvate, as well as the TCA (tricarboxylic acid) cycle intermediate oxaloacetate, in human glioblastoma cells. We show that these endogenously occurring 2-oxoacid analogues of 2-oxoglutarate can effectively stabilize HIF-1α and activate HIF-dependent gene expression. Pyruvate and oxaloacetate also prominently increase mRNA and protein levels of HPH-1 and HPH-2 independently of hypoxia. Glycolytic and TCA cycle metabolites may thus regulate a feedback loop featuring HIF and HPH homologues independently of hypoxia.

EXPERIMENTAL

Cell culture and hypoxia treatments

The human glioblastoma U87, U251 and U373 cell lines were cultured in Eagle's minimum essential medium with Earle's balanced salt solution without L-glutamine, supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. Cell lines were maintained in 5 % CO_2/95 % air atmosphere at 37 °C. For hypoxia treatments, the culture dishes were sealed in an incubator chamber and flushed with a gas mixture of 1 % O_2/5 % CO_2/94 % N_2 for 5 min, and incubated under this environment at 37 °C for the indicated time.

Materials

Culture media and fetal bovine serum were from Mediatech. DMOG was from Frontier Scientific. All remaining chemicals were from Sigma. The pcDNA3.1/V5-HIS human VHL vector was a kind gift from Dr Richard Bruick and Dr Steven McKnight (Graduate School of Biomedical Sciences, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.).

Antibodies

Mouse monoclonal anti-HIF-1α (610959) antibody was obtained from Transduction Laboratories. Polyclonal antibodies against HPH-1 (NB 100-139) and HPH-2 (NB 100-137) were purchased from Novus Biologicals. Mouse monoclonal antibody against β-actin (ab6276) was purchased from Abcam. Horseradish-peroxidase-coupled sheep anti-mouse antibody and mouse anti-rabbit antibody were obtained from Amersham Biosciences.

HIF-1α immunocytochemisty

Cells were seeded in 48-well plates and treatments were performed in glucose-free Krebs–Henseleit buffer (1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 124 mM NaCl, 3.5 mM KCl, 1.25 mM K_2HPO_4 and 26.3 mM $NaHCO_3$, pH 7.5). All drugs were dissolved in glucose-free Krebs–Henseleit buffer. Treatments were performed as indicated and then cells were washed in cold PBS, fixed (10 % formalin for 20 min) and permeabilized (0.2 % Triton X-100). Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and 0.5 % normal horse serum in PBS. Cells were incubated overnight at 4 °C with anti-HIF-1α antibody at 1:200 in PBS with 0.3 % Triton X-100 and 1.5 % normal horse serum. After washing with cold PBS, primary antibody was detected using ABC elite kit with biotinylated horse anti-mouse secondary antibody at 1:200 (Vector Laboratories). Immunoreactions were visualized with 3,3-diaminobenzidine.

Western blot analysis of HIF-1α, HPH-1 and HPH-2

For HPH-1 and HPH-2, whole cell extracts were prepared by lysing pelleted cells in RIPA buffer (0.1 % SDS, 1 % Nonidet P40, 5 mM EDTA, 0.5 % sodium deoxycholate, 150 mM NaCl, 50 mM Tris/HCl, freshly supplemented with 2 mM dithiothreitol and protease inhibitors) for 30–60 min on ice. Lysates were centrifuged at 16 000 ***g*** for 10 min (4 °C), the supernatant was subjected to electrophoresis in Novex 4–12 % Tris/glycine precast gels (Invitrogen), transferred on to nitrocellulose, and blotted using the polyclonal antibodies as described above. Nuclear extracts and Western blotting for HIF-1α was performed as previously described [21].

RT (reverse transcriptase)-PCR and quantificative RT-PCR analysis of oxygen sensors in various tissues and human glioma cell lines

Total RNA from cell lines were prepared using the RNeasy Mini Kit (Qiagen). Total RNA from human brain, heart and testis tissue was purchased (ClonTech). Total RNA (1.8 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The following forward/reverse primers were used: for human HPRT1 (hypoxanthine phosphoribosyltransferase 1) (GenBank® accession no. NM_000194), 5′-TGACACTGGCAAAACAATGCA-3′/5′-GGTCCTTTTCACCAGCAAGCT-3′; for human HPH-1/EGLN3 (see the Results section for an explanation of the EGLN nomenclature) (GenBank® accession no. NM_022073), 5′-AGATGTGGAGCCCATTTTTG-3′/5′-CAGATTTCAGAGCACGGTCA-3′; for human HPH-2/EGLN1 (GenBank® accession no. NM_022051), 5′-AAACCATTGGGCTGCTCAT-3′/5′-CGTACATAACCCGTTCCATTG-3′; for human HPH-3/EGLN2 (GenBank® accession no. NM_053046), 5′-CTGCTGCAGATCTTCCCTGAG-3′/5′-TAGGCGGCTGTGATACAGGT-3′; for human FIH-1 (GenBank® accession no. NM_017902), 5′-AACTGGGCCTACAAGCTCAA-3′/5′-CTTGCACCCCTAGTGTGGAT-3′; for human CA9 (carbonic anhydrase IX) (GenBank® accession no. NM_001216): 5′-CACTCCTGCCCTCTGACTTC-3′/5′-AGAGGGTGTGGAGCTGCTTA-3′. HotStarTaq DNA Polymerase (Qiagen) was used for RT-PCR amplifications. For quantificative real-time PCR analysis, the SYBR Green PCR Master Mix (PerkinElmer) and ABI Prism 7700 Detection System was used. Single-band amplification was verified through multicomponent analysis.

HIF peptide hydroxylation assay

U251 cell cytoplasmic extract was made by harvesting cells in lysis buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ and 1 mM dithiothreitol) at 4 °C using a Dounce homogenizer. The lysate was centrifuged at 20 000 ***g*** for 15 min. The supernatant (200 μg) was used as the source of enzyme in the ^{35}S-labelled pVHL pulldown assay, as described previously by Bruick et al. [8].

U251 cell HRE (hypoxia-response element)–luciferase assay

U251 cells stably expressing a luciferase reporter gene under the control of three copies of HRE (a gift from Dr Giovanni Melillo, National Cancer Institute-Frederick, Frederick, MD, U.S.A.) were cultured in 12-well plates. Luciferase assays were performed in a 96-well microtiter plate luminometer (Dynex Technologies) using the Luciferase Assay System (Promega). Each treatment was carried out in triplicate.

RESULTS

Human glioma cells express HIF hydroxylases and degrade HIF-1α more rapidly after prolonged hypoxia

Three different nomenclatures are currently used for the three identified human homologues of HIF prolyl hydroxylases: the HPH nomenclature reflects the unique HIF prolyl hydroxylase

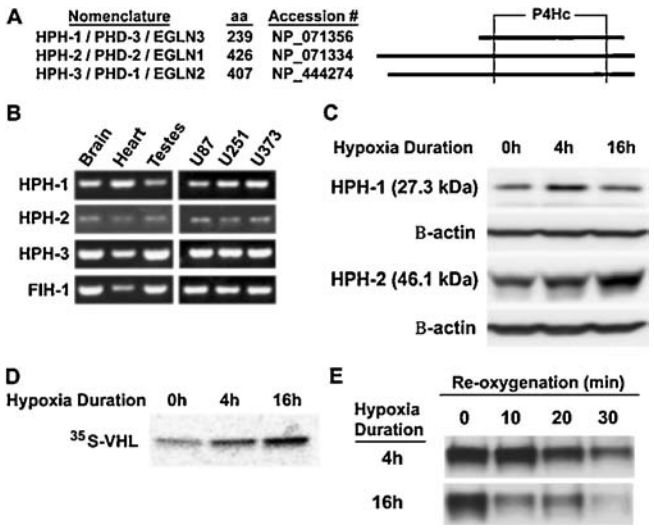


Figure 1 Human glioma cells express inducible oxygen sensors

The expression of oxygen sensors in human glioma cells was studied by PCR analysis, HIF-1α protein decay, HIF-1α ODD hydroxylation activity and Western blotting. (**A**) Schematic depiction of HPH homologues with their respective nomenclatures, amino acid content, GenBank® accession numbers and relative position of the consensus prolyl 4-hydroxylase domain (P4Hc). (**B**) PCR amplification of HPH homologues and FIH-1 from human glioma cDNA as compared with cDNA from human brain, heart and testis. (**C**) Hypoxia increases HPH protein levels. Cell extracts were prepared from control cells grown in 21 % O_2 (0 h) or from cells pretreated for 4 h and 16 h with 1 % O_2. Western blot analysis of the U251 extracts shows increased immunoreactivity for HPH-1 and HPH-2. β-Actin was used as a loading control. (**D**) Induction of HIF prolyl hydroxylase activity by hypoxia in U251 cells. Cell extract (200 μg) prepared in (**C**) was used for proline hydroxylation of an immobilized HIF-1α peptide, and was assayed by the ^{35}S-labelled pVHL pull-down assay, as described in the Experimental section. Extracts from cells exposed to increasing duration of hypoxia displayed increasing HIF-1α-peptide-hydroxylating activity. (**E**) Enhancement of HIF-1α decay rate following prolonged periods of hypoxia. U251 cell nuclear extracts were analysed by Western blotting for HIF-1α protein at different re-oxygenation times, as indicated, following either 4 h or 16 h exposure to 1 % O_2.

activity of these enzymes and is the nomenclature used here. The PHD nomenclature reflects the presence in these enzymes of a consensus prolyl hydroxylase domain that is found in other prolyl hydroxylases, such as those involved in collagen synthesis [7]. The EGLN nomenclature reflects the homology of these enzymes to the *Caenorhabditis elegans* egg-laying defect gene, EGL-nine [7]. The relationship between these nomenclatures is such that HPH-1, HPH-2, HPH-3 $\equiv$ PHD-3, PHD-2, PHD-1 $\equiv$ EGLN-3, EGLN-1, EGLN-2 respectively (Figure 1A).

Mammalian tissues display unique expression profiles for HPH mRNAs. Using specific PCR primers we identified mRNA for all 3 HPH homologues, as well as FIH-1, in each of the U87, U251 and U373 human glioma cells lines (Figure 1B). The relative expression profile of these genes was similar among the glioma cell lines, and more similar to that of human brain than of heart or testis [15] (Figure 1B). Both HPH-1 and HPH-2 have recently been shown to be up-regulated by hypoxia [7,13,16,17]. Using polyclonal antibodies against HPH-1 and HPH-2, we also observed enhanced expression of the HPH-1 and HPH-2 proteins following hypoxia (1 % O_2) for 4 h and 16 h, as compared with normoxic (21 % O_2) controls (Figure 1C). Using the same glioma cell extracts that displayed enhanced HPH-1 and HPH-2 protein expression, we also observed a greater enhancement of HIF-1α ODD peptide hydroxylating activity in extracts from U251 cell exposed to 16 h of hypoxia compared with those exposed to only 4 h of hypoxia (Figure 1D). HPHs promote proteolytic decay of HIFα subunits. HeLa cervical carcinoma cells have been shown to display an acceleration of HIF-1α decay following prolonged hypoxia [18]. Similarly, we found that in human U251 glioma cells, the rate of HIF-1α decay upon re-oxygenation was faster following 16 h of exposure to hypoxia (1 % O2) than following 4 h of hypoxia (Figure 1E). Thus human glioma cells express inducible HPH homologues.

2-Oxoacid metabolites of glycolysis and the TCA cycle can activate HIF

The HIF pathway can also be activated by several stimuli under normoxia [22]. We recently employed short-term culture of human glioma cells in Krebs buffer to identify pyruvate as a key glucose metabolite that could increase HIF-1α protein levels [21]. To distinguish the contribution of glucose metabolites from other stimuli in HIF activation, we cultured glioma cells for several hours in glucose-free Krebs buffer with selective additions (Figure 2). The glioma cell lines grown in this manner were found to be viable for at least 24 h and were capable of robust HIF activation. This was demonstrated using Western blotting to assess HIF-1α protein stabilization in both U87 and U251 cells following 4 h exposure to hypoxia, DFO, cobalt or DMOG (Figure 2A). Similar to these well-known stimulators of HIF-1, we found that pyruvate and oxaloacetate, two endogenous 2-oxoacid analogues of 2-oxoglutarate that are derived from glycolysis and the TCA cycle respectively, were also efficient inducers of HIF-1α protein stability (Figure 2A). Other TCA cycle intermediates that occur between pyruvate and oxaloacetate were without effect ([21], and results not shown). Similar to hypoxia and the other hypoxia-mimicking agents, pyruvate and oxaloacetate also clearly promoted HIF-1α nuclear translocation (Figure 2B), as well as the expression of a luciferase construct under the control of a HIF-regulated promoter (Figure 2C). 2-Oxoglutarate itself did not promote HIF-1α accumulation (Figure 2B). Thus, similar to pyruvate, the TCA cycle intermediate oxaloacetate can specifically promote HIF-1α stability and HIF-1 transactivational activity independently of hypoxia.

Pyruvate and oxaloacetate differentially induce expression of oxygen sensors in glioma cells

To evaluate the contribution of hypoxic and normoxic HIF-1α activators in regulating the expression of HIF-1α hydroxylating enzymes, we cultured U251 glioma cells in glucose-free Krebs for 8 h and individually determined the effects of hypoxia, hypoxia-mimicking agents, pyruvate and oxaloacetate on the expression of HPH-1, HPH-2, HPH-3 and FIH-1 mRNA by using quantificative real-time RT-PCR analysis. As shown in Figure 3(A), hypoxia, DFO, cobalt, DMOG, pyruvate and oxaloacetate all markedly enhanced the expression of HPH-1 and HPH-2, but not HPH-3 or FIH-1. As expected the expression of CA9, a well-known HIF-1 regulated gene, was also upregulated by these stimuli. The enhancement of HPH-1 and HPH-2 by all stimuli, as shown for pyruvate in Figure 3(B), was inhibited by actinomycin D. Moreover, protein levels of both HPH-1 and HPH-2 were upregulated by each of these treatments (Figure 3C). These studies were performed in glucose-free buffers in order to eliminate substantial contribution to HIF-1 activation via a build-up of glycolytic metabolites. To confirm that glucose metabolism could indeed influence expression of HPH homologues, we also employed an alternate pharmacological strategy. U251 cells were transferred from medium into Krebs buffer with or without 20 mM glucose. A significantly higher expression level of HPH-1 and HPH-2 was seen after 8 h culture in the glucose-containing buffer, which was blocked by the presence of 5 μM iodoacetate (Figure 4). The inhibitory effect of iodoacetate could, however, be bypassed

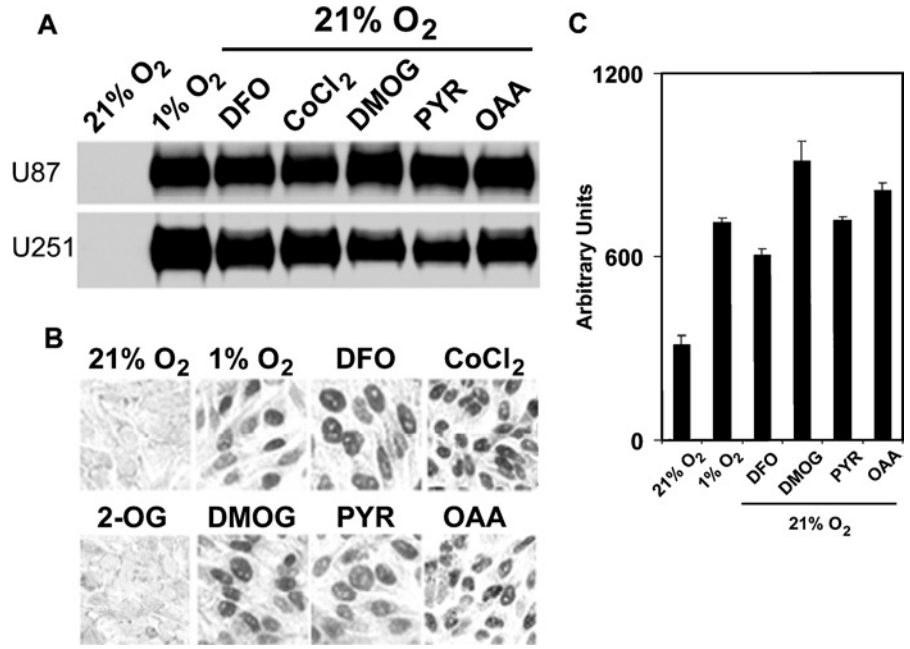


Figure 2 Normoxic activation of HIF-1 by endogenous 2-oxoacids

In order to independently examine HIF-1 activation by hypoxia, hypoxia mimetics and glucose-derived metabolites, U87 and U251 cells were switched from their culture media to glucose-free Krebs–Henseleit buffer. (**A**) Cells were treated with either hypoxia (1 % O_2) or the indicated stimuli under normoxia (21 % O_2). Following 4 h treatment, cells were evaluated for nuclear HIF-1α levels by Western blot analysis. PYR, pyruvate; OAA, oxaloacetate. (**B**) Direct nuclear accumulation of HIF-1α levels was monitored in U251 cells by immunocytochemsitry after treating cells with 1 % O_2, or the indicated stimuli under normoxia in glucose-free Krebs–Henseleit buffer for 4 h. 2-OG, 2-oxoglutarate. (**C**) Increases in HIF-1-mediated gene expression was monitored in U251 cells stably transfected with HRE–lucerifase reporter construct. Cells in 12-well plates were switched to glucose-free Krebs–Henseleit buffer, and treated with 1 % O_2 or the indicated stimuli under normoxia for 4 h. Doses used: DFO, 150 μM; $CoCl_2$, 100 μM; DMOG, 1 mM in (**A**) and (**B**), and 0.5 mM in (**C**); pyruvate, 2 mM; oxaloacetate, 2 mM. Experiments were performed in triplicate and repeated at least twice. Results from all treatments were statistically significant with respect to 21 % O_2 ($P < 0.01$, Student's t test).

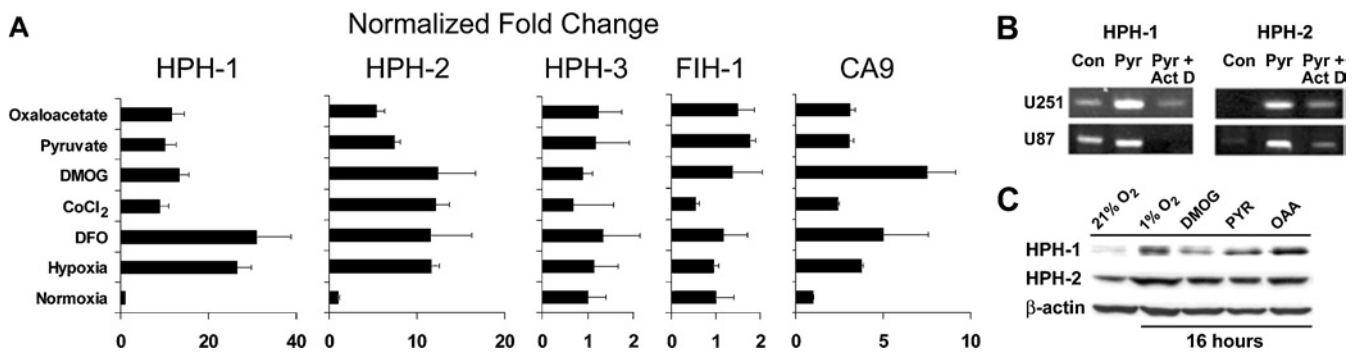


Figure 3 Differential regulation of oxygen sensor mRNA expression by hypoxia, hypoxia mimetics and endogenous 2-oxoacids

(**A**) Cells were cultured as described earlier, switched to glucose-free Krebs–Henseleit buffer, and treated with 1 % O_2, or the following agents in normoxia for 8 h: 150 μM DFO, 100 μM $CoCl_2$, 1 mM DMOG, 2 mM pyruvate or 2 mM oxaloacetate. The level of mRNA was determined by quantificative RT-PCR using the SYBR green dye, as described in the Experimental section. The amount of each mRNA in samples was normalized to the HPRT1 mRNA in the same sample. Genomic amplification was minimized by using primers that span exons and by verifying single amplicons through multicomponent analysis. Results are represented as the fold change of normalized level of mRNA in the treatment over the value obtained in normoxic controls. The mean from at least four independent experiments is shown. For HPH-1, HPH-2 and CA9, results from all treatments were statistically significant with respect to normoxia ($P < 0.01$, Student's t test). (**B**) U251 and U87 cells were switched to Krebs–Henseleit buffer and treated with 2 mM pyruvate (Pyr) for 8 h in the presence or absence of 0.5 μg/ml of actinomycin D (Act D), as indicated. mRNA levels of HPH-1 and HPH-2 were analysed by RT-PCR and compared with untreated controls (Con). A representative experiment, out of two, is shown. (**C**) U251 cells were switched to Krebs–Henseleit buffer and treated with 1 % O_2, or the following agents in normoxia for 16 h: 1 mM DMOG, 2 mM pyruvate (PYR) or 2 mM oxaloacetate (OAA). Western blot analysis was performed as described in the Experimental section.

by the inclusion of 2 mM pyruvate or oxaloacetate in the culture buffer.

DISCUSSION

The main novel data presented in the present study are that oxaloacetate, a TCA cycle metabolite, can activate HIF, and that both pyruvate and oxaloacetate can also induce expression of HPH-1 and HPH-2 genes. HIF prolyl and asparaginyl hydroxylases are now recognized as mammalian oxygen sensors that belong to the large family of oxygen-, iron- and 2-oxoglutarate-dependent dioxygenases [7,8,11]. HIF hydroxylase activity can be inhibited by lack of oxygen and displacement of iron or 2-oxoglutarate. This observation explains the ability of several

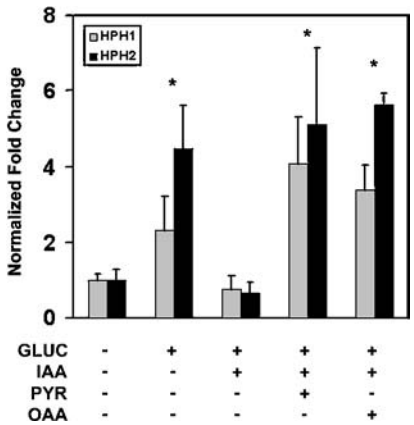


Figure 4 Regulation of oxygen sensor mRNA expression by glycolysis is inhibited by the glycolysis inhibitor, iodoacetate, and restored by endogenous 2-oxoacids

Cells were cultured as described earlier, switched to glucose-free Krebs–Henseleit buffer, and were treated with the following agents in normoxia for 8 h, as indicated: 20 mM glucose (GLUC), 5 μM iodoacetate (IAA), 2 mM pyruvate (PYR) and/or 2 mM oxaloacetate (OAA). The level of mRNA was determined by quantificative RT-PCR using the SYBR green dye, as described in the Experimental section. The amount of each mRNA in samples was normalized to the HPRT1 mRNA in the same sample. Genomic amplification was minimized by using primers that span exons and by verifying single amplicons through multicomponent analysis. Results are represented as the fold change of normalized level of mRNA in the treatment over the value obtained in normoxic controls. The means $\pm$ S.D. for three independent experiments are shown. For all treatments, results were statistically significant with respect to the normoxia and glucose plus iodoacetate (IAA) treatments ($P < 0.05$, Student's t test).

mimickers of hypoxia to activate HIF. Altered gene expression of specific HPH homologues is a newly appreciated means of regulating hypoxic transcriptional responses. So far, the HPH-1 and HPH-2 homologues are known to be inducible genes with hypoxia being the only known physiological stimulus capable of their induction [7,13,16,17]. HIF-1 is strongly implicated in the hypoxic induction of HPH homologues, thus suggesting a novel feedback loop for blunting hypoxic gene expression. In the present work, we have confirmed the existence of such a feedback mechanism in human glioma cells. We have demonstrated that hypoxia up-regulates mRNA, protein and enzymic activity of HPH-1 and HPH-2. Increased HIF prolyl hydroxylation activity with increasing duration of hypoxia may underlie the faster rate of HIF-1α proteolysis seen upon re-oxygenation (Figure 1). This may represent a mechanism for limiting hypoxia-inducible gene expression and also for adapting to re-oxygenation. Additionally, changes in the relative expression profile of oxygen sensors may be of physiological relevance, since the three HPHs have differing affinity for HIFα subunits [23,24] and individual HPH homologues may have different activities toward the two target proline residues on various HIFα subunits [7,23]. Differential induction of HPH homologues may thus alter the relative cellular ratios of HIFα subunit isoforms.

Although hypoxia is the ubiquitous inducer of HIF-1α in all cells tested, other physiological stimuli, such as cytokines, growth factors, reactive oxygen species, nitric oxide and pyruvate, can also activate HIF under normoxic conditions [20,21]. The mechanism of action for most of these normoxic HIF activators remains unclear and only nitric oxide has been shown to directly inhibit HIF prolyl hydroxylases [25]. We have shown here that the endogenous 2-oxoacids pyruvate and oxaloacetate can both effectively stabilize HIF-1α and activate HIF-dependent gene expression (Figure 2). These results provide the first evidence for regulation of the HIF-1 pathway by oxaloacetate. Although similar to pyruvate, oxaloacetate is a key intermediate at the crossroads of several metabolic pathways, it plays unique biochemical roles in transferring reducing equivalents between the mitochondrial and cytoplasmic compartments, and also serves as the key intermediate in gluconeogenesis. Both pyruvate and oxaloacetate have been shown to inhibit some 2-oxoglutarate-dependent dioxygenases [26], but not others [27]. By acting as endogenous antagonists for HIF hydroxylases, pyruvate and oxaloacetate may constitute a hypoxia-independent regulation of HIF-mediated gene expression, which responds to changes in cellular energy metabolism and nutritional status. Moreover, inhibition of mitochondrial oxidative metabolism during hypoxia could lead to elevated levels of these 2-oxoacids, thus suggesting a collaborative role for these metabolites in hypoxic signalling.

Anaerobic energy metabolism is prominently stimulated by HIF activation via the induction of several genes for glucose transporters and glycolytic enzymes [28–30]. The hypoxia-induced up-regulation of HIF which elevates levels of anaerobic metabolites, such as pyruvate and oxaloacetate, could act in a feedforward manner to sustain HIF activation, even upon re-oxygenation. We postulated that, as with hypoxia, normoxic HIF activators may also enhance the expression of HPH homologues to prevent such a feedforward cycle. Like hypoxia, both pyruvate and oxaloacetate were found to up-regulate HPH-1 and HPH-2 mRNA levels in cell culture (Figure 3). Furthermore, inhibition of ongoing glycolytic metabolism by iodoacetate inhibited the expression of HPH-1 and HPH-2. This provides the first evidence for transcriptional regulation of HPH homologues by physiological stimuli other than oxygen. Regulation of HPH gene expression by glycolytic and TCA cycle metabolites could independently modify the oxygen-sensing abilities of cells, and may underlie important interactions between oxygen homoeostasis and glucose metabolism [29,30]. Intermediates of cellular energy metabolism may also influence the newly identified actions of HPH homologues in regulating cell growth control [31,32].

This work was supported by National Institutes of Health grant NS-37814, U.S. Department of Defense grants MDA905-92-Z-0003 and MDA905-00-1-0034, and a grant from the Mary Kay Ash Charitable foundation to A. V. The views and opinions asserted herein are those of the authors and are not to be construed as representing the opinions of the U.S. Department of Defense.

REFERENCES

1 Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer **3**, 721–732
2 Wang, G. L. and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. **270**, 1230–1237
3 Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin–proteasome pathway. Proc. Natl. Acad. Sci. U.S.A. **95**, 7987–7992
4 Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L. (1999) Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin–proteasome pathway. J. Biol. Chem. **274**, 6519–6525
5 Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (London) **399**, 271–275
6 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-α to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science (Washington, D.C.) **292**, 468–472
7 Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A. et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell (Cambridge, Mass.) **107**, 43–54
8 Bruick, R. K. and McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science (Washington, D.C.) **294**, 1337–1340

9 Sang, N., Fang, J., Srinivas, V., Leshchinsky, I. and Caro, J. (2002) Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1α is governed by a von Hippel–Lindau protein-independent, hydroxylation-regulated association with p300/CBP. Mol. Cell. Biol. **22**, 2984–2992
10 Mahon, P. C., Hirota, K. and Semenza, G. L. (2001) FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. **15**, 2675–2686
11 Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. **16**, 1466–1471
12 McNeill, L. A., Hewitson, K. S., Claridge, T. D., Seibel, J. F., Horsfall, L. E. and Schofield, C. J. (2002) Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the β-carbon of asparagine-803. Biochem. J. **367**, 571–575
13 Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W. et al. (2003) Intracellular localisation of human HIF-1α hydroxylases: implications for oxygen sensing. J. Cell Sci. **116**, 1319–1326
14 Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R. and Taubman, M. B. (2002) Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. Biochem. Cell Biol. **80**, 421–426
15 Oehme, F., Ellinghaus, P., Kolkhof, P., Smith, T. J., Ramakrishnan, S., Hutter, J., Schramm, M. and Flamme, I. (2002) Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem. Biophys. Res. Commun. **296**, 343–349
16 Naranjo-Suarez, S., Castellanos, M. C., Alvarez-Tejado, M., Vara, A., Landazuri, M. O. and del Peso, L. (2003) Down-regulation of hypoxia-inducible factor-2 in PC12 cells by nerve growth factor stimulation. J. Biol. Chem. **278**, 31895–31901
17 del Peso, L., Castellanos, M. C., Temes, E., Martin-Puig, S., Cuevas, Y., Olmos, G. and Landazuri, M. O. (2003) The von Hippel–Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. J. Biol. Chem. **278**, 48690–48695
18 Berra, E., Richard, D. E., Gothie, E. and Pouyssegur, J. (2001) HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1α degradation. FEBS Lett. **491**, 85–90
19 Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D. and Pouyssegur, J. (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. **22**, 4082–4090
20 Bilton, R. L. and Booker, G. W. (2003) The subtle side to hypoxia inducible factor (HIFα) regulation. Eur. J. Biochem. **270**, 791–798
21 Lu, H., Forbes, R. A. and Verma, A. (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem. **277**, 23111–23115
22 Chun, Y. S., Kim, M. S. and Park, J. W. (2002) Oxygen-dependent and -independent regulation of HIF-1α. J. Korean Med. Sci. **17**, 581–588
23 Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I. and Myllyharju, J. (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J. Biol. Chem. **278**, 30772–30780
24 Huang, J., Zhao, Q., Mooney, S. M. and Lee, F. S. (2002) Sequence determinants in hypoxia-inducible factor-1α for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. J. Biol. Chem. **277**, 39792–39800
25 Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J. and Brune, B. (2003) Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. Mol. Biol. Cell **14**, 3470–3481
26 Ng, S. F., Hanauske-Abel, H. M. and Englard, S. (1991) Cosubstrate binding site of *Pseudomonas* sp. AK1 γ-butyrobetaine hydroxylase: interactions with structural analogs of α-ketoglutarate. J. Biol. Chem. **266**, 1526–1533
27 Kaule, G., Timpl, R., Gaill, F. and Gunzler, V. (1998) Prolyl hydroxylase activity in tissue homogenates of annelids from deep sea hydrothermal vents. Matrix Biol. **17**, 205–212
28 Semenza, G. L., Roth, P. H., Fang, H. M. and Wang, G. L. (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem. **269**, 23757–23763
29 Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K. and Johnson, R. S. (2001) Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol. Cell. Biol. **21**, 3436–3444
30 Minchenko, A., Leshchinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V. and Caro, J. (2002) Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene: its possible role in the Warburg effect. J. Biol. Chem. **277**, 6183–6187
31 Erez, N., Milyavsky, M., Eilam, R., Shats, I., Goldfinger, N. and Rotter, V. (2003) Expression of prolyl-hydroxylase-1 (PHD1/EGLN2) suppresses hypoxia inducible factor-1α activation and inhibits tumor growth. Cancer Res. **63**, 8777–8783
32 Frei, C. and Edgar, B. A. (2004) *Drosophila* cyclin d/cdk4 requires hif-1 prolyl hydroxylase to drive cell growth. Dev. Cell **6**, 241–251

Received 28 October 2003/20 February 2004; accepted 25 February 2004
Published as BJ Immediate Publication 25 February 2004, DOI 10.1042/BJ20031647

SUMMARY OF PAPER 2

Rationale: We recently showed that in addition to hypoxia, HIF-1α protein levels and HIF-1 mediated gene expression are stimulated by the glycolytic metabolites pyruvate and oxaloacetate. The mechanism by which these glycolytic metabolites act on the HIF-1 signaling pathways is not clear. It is also unclear whether glycolytic HIF regulation has any physiological significance.

Objectives: In this paper, we set out to (a) determine whether pyruvate and oxaloacetate acted at the catalytic active site of the HPH prolyl hydroxylases due to their structural similarities to the main substrate of these oxygen sensors, 2-oxoglutarate; (b) determine whether pyruvate and oxaloacetate directly inhibited HIF hydroxylation; (c) determine whether HIF-1 induction by glycolytic metabolites contributed to HIF-1 mediated malignant features of cancer cells.

Methods: HIF-1α protein levels were assessed by western blots in cells treated with a wide variety of glycolytic metabolites. Quantitative PCR analysis was used to examine expression of HIF-1 target genes. Affinity chromatography was used to determine binding interactions between HIF-1α and ^{35}S-pVHL and also between 2-oxoglutarate and ^{35}S-labelled HPH homologues. *In vitro* translated HPH homologues were used in to assess HIF hydroxylation enzymatic activity utilizing the ^{35}S-pVHL pulldown assay. HIF hydroxylation activity was also determined in cell extracts using this method. Cell invasion was assessed in Matrigel-coated (Boyden) chambers as a measure of HIF-regulated malignant phenotype.

Findings: Pyruvate and oxaloacetate were found to induce HIF in a manner distinct from that of hypoxia. Unlike hypoxia, these 2-oxoacids produced an inactivation of HIF hydroxylation activity. This inactivation was selectively reversed by ascorbate, histidine, cysteine, and glutathione. Oxidation of enzyme bound iron was implicated in the action of these 2-oxoacids. Inhibition of 2-oxoacid HIF-1α by ascorbate was correlated with an inhibition of cell invasiveness.

Significance: This study identified glucose-derived 2-oxoacids as novel regulators of HIF-1α via a mechanism that may be selectively targeted by ascorbate and other reducing agents. This study also elucidated a potential mechanism whereby aerobic glycolysis may promote malignant features in cancer cells.

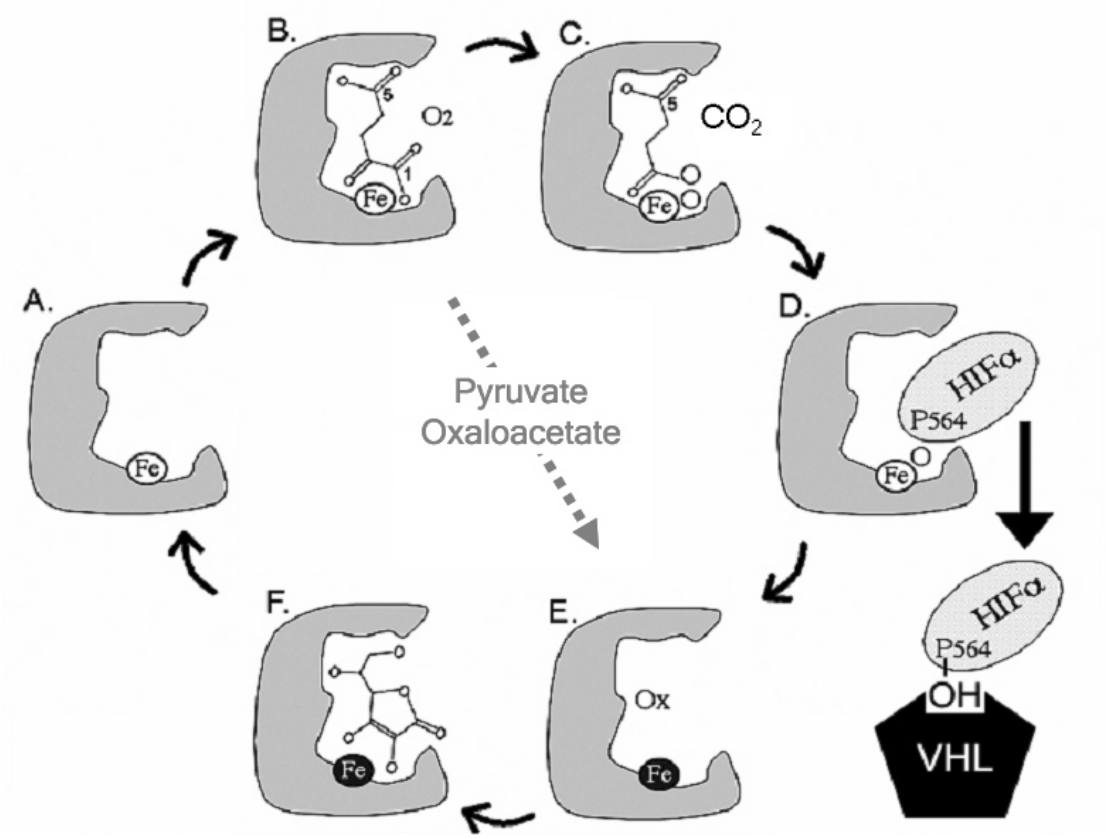


Figure 1. Putative enzymatic cycle for the HPH homologues.

Step A. Ferrous iron binding at the HPH active site. Step B. 2-Oxoglutarate (2-OG) and molecular oxygen (O_2) bind to the ferrous iron in the HPH active site. Step C. Oxidative decarboxylation of 2-OG into succinate and CO_2. Step D. Binding and hydroxylation of the HIF-1α at a proline residue in the oxygen-dependent degradation domain. Step E. Ferrous iron in the HPH active site is oxidized. Step F. Reactivation of HPH and iron by ascorbate. The presence of pyruvate or oxaloacetate may lead to a futile reaction in the enzymatic cycle, causing an oxidation of the ferrous iron in the active site without an accompanying hydroxylation of HIF-1α proline residue. A shift in the ratio of futile reactions to hydroxylation reactions may lead to the build up of HIF-1α protein levels in the cell and HIF-1 activation of gene expression.

(Submitted to Cancer Cell in fulfillment of requirements for degree)

REVERSIBLE INACTIVATION OF HIF PROLYL HYDROXYLASES BY ENDOGENOUS 2-OXOACIDS ALLOWS HIF-1 TO SERVE AS A METABOLIC SENSOR.

Clifton Dalgard[1], Huasheng Lu[1], Ahmed Mohyeldin[1], Thomas McFate, A. Sasha Tait, and Ajay Verma*.

Department of Neurology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD, 20814, Tel: 301-295-3840, Fax: 301-295-3825, averma@usuhs.mil

[1]These authors contributed equally to this work.

* To whom all correspondence should be addressed.

Supported by PHS Grant NS37814, and DOD grants (DBSCIP and USMCI) to A.V.

SUMMARY

Oxygen dependent hydroxylation of the HIF-1 transcription factor inhibits transcriptional responses to hypoxia. HIF-1α prolyl and asparaginyl hydroxylases are iron and 2-oxoglutarate-dependant dioxygenases that hydroxylate the HIF-1α subunit, thereby promoting its proteolysis and blocking its transactivation ability. Inhibition of HIF-1α hydroxylases during hypoxia allows HIF-1 to induce gene expression. Here we show that pyruvate and oxaloacetate induce HIF-1 mediated gene expression by a mechanism distinct from hypoxia. These 2-oxoacids bind to the 2-oxoglutarate site of HIF-1α prolyl hydroxylases and inactivate these enzymes in a manner selectively reversible by ascorbate, cysteine, histidine and ferrous iron but not by 2-oxoglutarate or oxygen. We show that inactivation of HIF hydroxylation by glucose-derived 2-oxoacids underlies the basal HIF-1 activity commonly seen in many cancer cells with high rates of aerobic glycolysis. Selective regulation of HIF-1 by endogenous 2-oxoacids may constitute a novel signaling mechanism involved in metabolic adaptation, ischemia and cancer.

Running Title:

Metabolic regulation of HIF-1.

INTRODUCTION

Mammalian cells adapt to hypoxia through the action of the heterodimeric transcription factor HIF-1. Such adaptations can also promote carcinogenesis by inducing angiogenesis, treatment resistance and invasiveness in hypoxic cancer cells within tumors (Semenza, 2003). In the presence of oxygen, the HIF-1α subunit is rapidly targeted to the ubiquitin-proteasome degradation pathway via the von Hippel-Lindau tumor suppressor gene product pVHL (Salceda and Caro, 1997; Huang et al., 1998; Maxwell et al., 1999). The binding of pVHL to HIF-1α requires the post-translational hydroxylation of proline residues (Pro402, Pro564) within the HIF-1α oxygen-dependent degradation (ODD) domain (Ivan et al., 2001; Jaakkola et al., 2001). This modification is prevented during hypoxia, thus allowing HIF-1α to escape proteolysis, dimerize with HIF-1β, and translocate to the nucleus. A separately controlled, O_2 dependent hydroxylation of asparagine 803 in the HIF-1α C-terminal transactivation domain (C-TAD) inhibits HIF-1 interaction with the p300/CBP coactivator, thereby blocking HIF-1 transcriptional activity in the presence of oxygen. (Lando et al., 2002; Hewitson et al., 2002).

Three HIF-1α prolyl hydroxylases (HPH 1-3; also referred to as PHD 3-1, respectively) and one O_2-dependant HIF-1α asparaginyl hydroxylase (Factor Inhibiting HIF; FIH) have been clearly identified so far (Bruick and McKnight, 2001; Epstein et al, 2001; Mahon et al., 2001). These enzymes are all members of the 2-oxoglutarate dependent family of dioxygenases and have an absolute requirement for oxygen, ferrous iron, and 2-oxoglutarate (2-OG). This explains

how hypoxia, iron chelators such as desferrioxamine (DFO) and artificial 2-OG analogs such as N-oxalylglycine (NOG) or its cell-permeable analog dimethyloxalylglycine (DMOG) can all prevent HIF-1α proteolysis and activate HIF-mediated gene expression. Ascorbate is also required for sustained activity of many 2-OG dependent dioxygenases (Schofield and Ratcliffe, 2004; Hanauske-Abel and Popowicz, 2003).

Hypoxia-independent mechanisms also regulate HIF-1 and are highly relevant to the progression of human cancers since high levels of HIF-1α protein are seen not only in hypoxic tumor zones, but also in well-oxygenated tumor areas and metastatic nodules (reviewed in Semenza, 2003). In fact, many cancer cell lines cultured in room air (21% O_2) routinely display significant basal levels of HIF-1α protein as well as high basal expression of genes and biological activities controlled by HIF-1 (Zhong et al., 2002; Cohen et al., 2004). The mechanisms underlying basal HIF-1 expression in cancer have been highly pursued recently. Growth factors (e.g. insulin, IGF-1, EGF, heregulin) that stimulate the PI3K-Akt-mTOR pathways can activate HIF-1 in the presence of O_2 (reviewed in Semenza, 2003), as can reactive oxygen species (ROS) (Gerald et al., 2004) and nitric oxide (NO) (Metzen et al., 2003). We recently discovered that the accumulation of glycolytic metabolites such as pyruvate could also promote hypoxia-independent HIF-1 activation (Lu et al., 2002; Dalgard et al., 2004). Further support for a distinct oxygen-independent mechanism for HIF-1 regulation was recently provided by the observation that ascorbate selectively reverses basal

but not hypoxia induced HIF-1α accumulation (Knowles et al., 2003). Since any means of HIF-1 activation is associated with aggressive behavior in human cancers, we set out to identify the major determinants of hypoxia-independent HIF-1 regulation in cancer cell lines.

RESULTS:

Specific metabolic fuels support hypoxia-independent HIF-1α levels.

Serum is a major source of growth factors and the main source of iron in cell culture, while media provides metabolic fuels. When we examined basal HIF-1α levels in several cancer cell lines cultured in media with widely varying glucose concentrations but identical serum supplementation, we observed that ascorbate reversible basal HIF-1α accumulation was higher in cell lines grown in high glucose DMEM (25mM) than in RPMI (11.1mM) or MEM (5.5mM) (figure 1A). The U87 and U251 human glioblastoma cells cultured in MEM and displaying both basal and hypoxia-inducible HIF-1α accumulation were used to further explore the relationship between glucose metabolism and HIF-1α. When switched from their native MEM media to Krebs buffer for 4 hours, U251 cells showed no basal HIF-1α accumulation if glucose was replaced in the buffer by the non-glycolytic energy source glutamine (figure 1A). Despite the presence of 10mM glutamine, basal HIF-1α accumulation in U251 cells was dose-dependently increased by glucose.

2-oxoacid functional groups distinguish endogenous HIF-1α stabilizing metabolites.

Since glucose feeds into many metabolic pathways, we determined which cellular intermediary metabolites were capable of promoting HIF-1α accumulation when substituted for glucose in Krebs buffer. An extensive screen of all glycolytic, all tricarboxylic acid (TCA) cycle, and many amino acid metabolic intermediates revealed that while most metabolic substrate substitutions did not significantly alter cellular ATP levels over 4h (Supplemental Figure S1A), only a select group of 2-oxoacids were able to promote HIF-1α accumulation in U251 cells. Of these, only pyruvate and oxaloacetate were found to be universally active in all cancer cell lines used in this study. The branched chain 2-oxoacids α-ketoisocaproate, and α-keto-β-methylvalerate were also active in some but not all cell lines, and α-ketoisovalerate showed minor activity (Figure 1A). The 1-carboxylate and 2-oxo functional groups common to 2-OG and to the artificial HIF-1α inducers NOG and DMOG (Figure 1B) were also features of naturally occurring metabolic intermediates that induced HIF-1α (figure 1B). Succinate, alanine, pyruvaldehyde, malate, acetoacetate and β-hydroxybutyrate, all of which lack a 2-oxo group, were thus unable to induce HIF-1α accumulation in intact cells. However, several 2-oxoacids such as keto-malonate, α-ketobutyrate, and α-ketoadipate, which contained the 1-carboxylate and 2-oxo groups, did not induce HIF-1α accumulation in intact cells suggesting that molecular features opposite from the 2-oxoacid end of the molecule were also crucial for inducing HIF-1α. Phenylpyruvate, and fluoropyruvate, which are modified on the end opposite the 2-oxoacid group, were not able to induce HIF-1α accumulation while ethyl and methyl esters of pyruvate at the 1 position very effective. Although

lactate can induce HIF-1α accumulation, this requires conversion to pyruvate via lactate dehydrogenase (Lu, et al., 2002). In addition to inducing HIF-1α accumulation in a variety of human cancer cells, pyruvate and oxaloacetate were also found to induce HIF-1α accumulation in cultured normal human astrocytes as well as in normal rat brain when given via intraperitoneal injection (3g/Kg) to 14-day old male rats (Figure 1C). Cultured rat cerebrocortical neurons treated with pyruvate and oxaloacetate also accumulated HIF-1α protein and showed increased expression of the HIF-1 regulated gene heme oxygenase-1 (Figure 1C). Thus, the ability of 2-oxoacids to induce HIF-1 was not limited to neoplastic cells.

HIF-1 activation by 2-oxoacids is selectively reversed by ascorbate.

As with the basal HIF-1α expression of U87 and U251 glioma cells cultured in media, induction of HIF-1α by 3mM pyruvate or oxaloacetate in glucose-free Krebs buffer was also completely reversed by 100μM ascorbate (figure 1D). This effect was not reversed by 10mM 2-OG, and neither ascorbate or 2-OG could reverse HIF-1α induction by hypoxia or DMOG. Selective ascorbate blockade of pyruvate and oxaloacetate inducible HIF-1α accumulation was observed in all cells used in this study (data not shown). Using U251 cells stably expressing a hypoxia regulatory element (HRE)-luciferase construct (U251-HRE cells; figure 1D), we found that pyruvate and oxaloacetate induced the HIF-1 regulated reporter gene as effectively as hypoxia or DMOG. Induction of the HIF-1 reporter gene by the 2-oxoacids was also selectively reversed by ascorbate, but not by 2-OG. Ascorbate did not reverse HRE-luciferase activation by hypoxia or by

DMOG, again suggesting a unique mode of HIF-1α regulation by pyruvate and oxaloacetate.

Cysteine, histidine, and glutathione antagonize glycolytic HIF induction. We observed that at an equivalent time in culture, HIF-1α expression in glioma cells tended to be higher in Krebs buffer than in complete culture media even when the glucose concentration was identical. Thus, when U87 glioma cells cultured in MEM were changed either to Krebs buffer or to fresh MEM with identical (5.5mM) glucose concentrations for 4h, a much higher induction of HIF-1α was always seen in Krebs buffer (Figure 2A). Since neither Krebs buffer nor the complete MEM media contained ascorbate, this suggested to us that complete MEM contained some inhibitory activity that prevented glycolytic activation of HIF-1α. MEM differs from Krebs buffer by its vitamin and amino acid additives. Upon reconstitution of Krebs buffer with these additives, we discovered that the amino acid mixture, and not the vitamin mixture, specifically blocked basal HIF-1α accumulation (Figure 2A). The amino acid mixture also blocked the buildup of HIF-1α induced by pyruvate in cells cultured for 4h in glucose-free Krebs. By reconstituting Krebs buffer with one essential amino acid at a time at the concentration normally present in MEM we determined that cysteine and histidine were responsible for inhibiting basal HIF-1α accumulation (figure 2B). This determination was reached following evaluation of all naturally occurring amino acids, only some of which are presented. Increasing the levels of each amino acid to 5x its normal level in MEM also revealed that cysteine and histidine were the likely endogenous inhibitors of basal HIF-1α accumulation in culture

media. Both cysteine and its dimer cystine (Cys2) were effective, while the D-cysteine and D-histidine were not (Figure 2C). Both amino acids lowered HIF-1α induction by glucose, pyruvate, and oxaloacetate in all cell lines used in this study (data not shown) but did not affect HIF-1α accumulation by hypoxia or the iron chelator DFO (Figure 2C). Cysteine is involved in glutathione (GSH) synthesis and as with ascorbate, cysteine, and histidine, GSH selectively inhibited HIF-1α accumulation induced by pyruvate and oxaloacetate but not hypoxia or DFO (Figure 2C). Increasing the levels of cysteine and histidine, but not other amino acids, to 5x their normal level in MEM, also lowered the basal expression of the HIF-1 regulated genes VEGF and GLUT3 as determined by RT-PCR (Figure 2D). Similar results were seen for U251 cells (data not shown). Cysteine and histidine also lowered HRE-luciferase activity induced by pyruvate or oxaloacetate in the U251-HRE cells (figure 2D). These data provide evidence for both positive and negative oxygen-independent regulation of basal HIF-1 expression by cell metabolites.

Pyruvate and oxaloacetate impede oxygen dependent protein decay.

Since ascorbate, cysteine, histidine and glutathione all have antioxidant and/or iron reducing properties, we next determined whether enhanced production of oxidants such as ROS or NO could underlie HIF-1 induction by pyruvate or oxaloacetate. Despite confirming the ability of H_2O_2 and NO to induce HIF-1α accumulation in U251 cells, we detected no increase in H_2O_2 or NO levels upon treatment of U251 cells with pyruvate or oxaloacetate (Figure 3A). Pharmacological inhibition of other signaling pathways implicated in HIF-1α

stabilization, such as protein acetylation, PI3K, mTOR, and hsp70, also failed to alter HIF-1α induction by pyruvate and oxaloacetate (Figure 3B). We next focused our attention on the action of HIF-1 hydroxylases as a target for 2-oxoacids. To explore this, we employed a live cell assay for oxygen-dependent HIF-1α degradation using C6 rat glioma cells that had been stably transfected with a vector encoding the HIF-1α C-terminal ODD region fused to GFP (ODD-GFP). The ODD-GFP protein is hydroxylated and degraded in an oxygen dependent manner similar to HIF-1α. As with HIF-1, these cancer cells display basal as well as hypoxia-inducible ODD-GFP (D'Angelo et al., 2002). We found that basal ODD-GFP accumulation in C6 cells, cultured in MEM media for 24h, was selectively inhibited by ascorbate, while the hypoxia inducible ODD-GFP expression was not (Figure 3C). In Krebs buffer, basal ODD-GFP accumulation was also stimulated by glucose, pyruvate, and oxaloacetate, but not by succinate. The ineffectiveness of succinate vs. pyruvate or oxaloacetate was not due to a relative difference in cell permeability, since the same pattern was seen in digitonin-permeabilized C6 cells (Supplemental Figure S1B). Moreover, histidine and cysteine also reduced ODD-GFP induction by pyruvate or oxaloacetate. These results suggested that endogenous 2-oxoacids blocked O_2 dependent protein degradation.

Pyruvate and oxaloacetate directly interact with HPHs.

Pyruvate does not inhibit proteasomal activity (Lu et al., 2002), and neither pyruvate nor oxaloacetate was found to inhibit ^{35}S-pVHL binding to hydroxylated ODD (Figure 4A). Artificial 2-OG analogs such as NOG promote HIF-1α

accumulation by strongly competing for the 2-OG binding site in HPHs (Epstein, et al., 2001). To determine if pyruvate and oxaloacetate were recognized by the HPH 2-OG binding site, we developed a binding assay using immobilized 2-OG and *in vitro* translated ^{35}S-HPH homologues. The observed binding of each homologue to the 2-OG column under our assay conditions was doubled by the addition of 200μM ferrous iron (Figure 4B). Moreover, the iron dependent binding of ^{35}S-HPHs to immobilized 2-OG was also readily displaced by addition of free 2-OG but not by succinate (Figure 4C). Pyruvate and oxaloacetate also reversed the iron dependent binding of ^{35}S-HPHs to immobilized 2-OG (Figure 4D), suggesting that these 2-oxoacids can interact with the HPH enzyme active site.

2-oxoacid inhibition of HPH activity is ascorbate-sensitive.

To determine whether HPH activity was directly modulated by pyruvate or oxaloacetate, we studied pro564 hydroxylation in a biotinylated HIF-1α ODD peptide by *in vitro* translated HPH1, HPH2, and HPH3. The ability of streptavidin beads to pull down ^{35}S-pVHL bound to the biotinylated peptide was used as evidence of HIF-1α ODD peptide hydroxylation. As exemplified by HPH-1, *in vitro* enzyme activity of each HPH homologue was dependent on 2-OG, Fe(ll), and ascorbate (Figure 4E). In the absence of ascorbate or other reducing agents, cysteine and histidine were also found to dose dependently stimulate HIF-1α ODD hydroxylation (Figure 4F). Some 2-OG dependent dioxygenases are able to accept pyruvate and oxaloacetate as substitutes for 2-OG (Kaule et al., 1998) while others are inhibited by these 2-oxoacids (Majamaa et al., 1985). When we replaced 2-OG in the reaction mix with identical concentrations of pyruvate or

oxaloacetate, no enzyme activity was detected for any of the three HPH homologues (Figure 4G). Instead, we detected inhibition of the 2-OG supported enzymatic activity by pyruvate and oxaloacetate for all three homologues. However, this inhibition was influenced by the concentration of ascorbate employed in the assay. Examples of raw data showing this effect are shown in Supplemental Figure S1C and D, and quantitative evaluation is shown in figure 4H. Inhibition of HPH2 and HPH3 by the 2-oxoacids was more potently reversed by ascorbate than HPH1. Ascorbate at 1mM reversed 2-oxoacid inhibition of all HPHs.

2-oxoacids reversibly inactivate cellular HPHs.

The ascorbate reversibility of pyruvate- and oxaloacetate-induced HIF-1α accumulation suggested to us that these 2-oxoacids could somehow inactivate cellular HPHs. To test this notion we exposed U251 cells in glucose-free Krebs buffer to either hypoxia (1% O_2) or 2mM pyruvate for 4h. Nuclear HIF-1α protein accumulation was induced by both treatments (Figure 5A). We then either returned the cells to 21% O_2 or removed the pyruvate by extensively washing with glucose-free Krebs buffer. HIF-1α immunoreactivity disappeared rapidly upon reoxygenation (return to 21% O_2) of hypoxic cells as would be expected with the resumption of oxygen-dependent HIF-1α hydroxylation and proteolysis. An entirely different pattern was seen in pyruvate treated cells. Even after extensive washing out of pyruvate, nuclear HIF-1α protein levels did not decay promptly. Persistent nuclear HIF-1α was also detected in pyruvate or oxaloacetate treated U87 cells, even 40 min after washout of either 2-oxoacid.

However, if ascorbate, GSH, or the essential amino acids found in MEM were added to the wash, HIF-1α decay was dramatically accelerated (Figure 5B). To directly determine if the HPH activity in glioma cells was reversibly inactivated by pyruvate and oxaloacetate we used the ^{35}S-pVHL pulldown assay to measure HPH activity in extracts from cells treated for 4h with the 2-oxoacids. To capture the endogenous state of enzyme activity, the cell extracts were first assayed without supplementation with exogenous reductants, ascorbate, or iron. Incubation of cells with 2mM pyruvate or oxaloacetate clearly reduced HPH activity in the cell extracts. This reduction was not seen in cells co-treated with ascorbate (Figure 5C). Moreover, no loss of HPH activity was seen in extracts from cells treated for 4h with hypoxia or DMOG, with or without ascorbate. The minimal HPH activity of extracts from pyruvate or oxaloacetate treated cells could also be restored by supplementation of the extracts with ascorbate or with exogenous ferrous iron ($FeSO_4$) during the assay (Figure 5D).

Oxidation of HPH associated iron from the Fe(II) to the Fe(III) state has recently been proposed as a mechanism for H_2O_2 regulation of HIF-1 in cells mutated for JunD (Gerald et al., 2004). We hypothesized that NO, another potent iron oxidizer, may also work this way and observed a complete inhibition of NO induced HIF-1α accumulation by ascorbate, Fe(II), and GSH (Figure 5E). Addition of Fe(II) to cultured cells also prevented HIF-1α induction by pyruvate and oxaloacetate. Moreover, NO treated cells also showed sluggish HIF-1α decay, even after extensive washing (Figure 5E) with MEM. Addition of Fe(ll) but not Fe(III) to the wash accelerated the decay of NO-induced HIF-1α. A similar

enhancement of HIF-1α decay by wash containing Fe(II) but not Fe(III) was seen following induction with pyruvate or oxaloacetate (Figure 5F). However, these 2-oxoacids do not share the direct iron oxidizing activity of H2O2 or NO, suggesting a more complex action at the enzyme active site. The need for ferrous iron for the decay of HIF-1α during the post-induction wash was also demonstrated using DFO. Chelation of cellular iron by this agent inhibits cellular HPH activity and prevents HIF-1α decay. After washing out the DFO, addition of Fe(ll) but not Fe(lll) promoted decay of the accumulated HIF-1α protein.

Aerobic glycolysis regulates basal HIF-1 activity and invasive phenotype in human cancer cells.

Even in the presence of oxygen, most cancer cells accumulate high levels of pyruvate and lactate (Warburg, 1930). As with hypoxia, high tumor lactate levels predict likelihood of metastases and poor clinical outcome in many human cancers (Walenta et al., 2004). Given the strong role of HIF-1 in cancer cell invasiveness (Semenza, 2003) we hypothesized that aerobic glycolysis derived 2-oxoacids could contribute to cancer cell invasiveness by inducing basal HIF-1α. To explore this notion we examined two head and neck squamous carcinoma (HNSSCA) cell lines known to have disparate basal HIF-1α levels (Cohen, et al, 2004). The JHU-SCC-022 cell line (022) was grown in RPMI containing 11.1mM glucose and the UM-SCC-22B cell line (22B) was grown in DMEM containing 25mM glucose. Media of both cell lines were supplemented equally with 10% FBS and the non-glycolytic energy source glutamine (2mM). Both cell lines demonstrated HIF-1α accumulation under hypoxia (Figure 6A). However, the

22B cells displayed much higher basal HIF-1α, elaborated higher basal levels of VEGF (Figure 6B), and had a higher basal rate of invasion through Matrigel than the 022 cells (Figure 6C). Serum, which activates many signaling pathways, markedly increased the invasiveness of both cell lines to a similar extent. When both cell lines were cultured in high glucose DMEM, 22B cells still consumed glucose faster and produced pyruvate and lactate at a higher rate than 022 cells (Figure 6 D-F). The temporal accumulation of basal HIF-1α in 22B cells required glucose but was not affected by the presence or absence of 10% FBS (Figure 6G). These results show that a high intrinsic rate of aerobic glycolysis was sufficient to account for basal HIF-1α accumulation. Cells cultured in DMEM, with or without glucose, were still able to prominently induce HIF-1 under hypoxia (Figure 6H). This not only showed that the cells could maintain prolonged viability in the presence of glutamine alone, but also clearly distinguished glycolytic and hypoxic regulation HIF-1 as separate mechanisms. We also observed an enhancement of HIF-1α accumulation in 22B cells upon pyruvate supplementation of DMEM and the addition of ferrous iron blunted this to a greater extent than ferric iron (Figure 6I). Ascorbate effectively reversed HIF-1α accumulation in the 22B cells when induced by pyruvate (Figure 6I) or by glucose but not by hypoxia or DMOG (Figure 6J,K). Ascorbate also lowered the basal expression of several HIF-1 regulated genes in 22B cells including carbonic anhydrase IX (CAIX), GLUT3, and matrix metalloprotease-2 (MMP-2) (Figure 6L). Furthermore, ascorbate lowered the basal invasiveness of 22B cells through Matrigel, without affecting serum-induced invasiveness (figure 6M). These results

implicate ascorbate-reversible, glycolysis-dependent basal HIF-1 activity in cancer progression.

DISCUSSION

HIF-1α hydroxylase activity governs the degradation of HIF-1α by oxygen. Investigations of hypoxia-independent HIF-1 regulation have therefore largely focused on mechanisms not involving these enzymes. We have elucidated here a novel mechanism by which 2-oxoacid metabolites can directly inactivate HIF-1α hydroxylation and degradation in a manner reversible by ascorbate, cysteine, histidine, and ferrous iron. HIF-1 can thus be up- and down- regulated by cellular metabolism, independently of oxygen. We propose that direct inactivation of HPHs by glucose-derived 2-oxoacids in cancer cells is a prominent mechanism for sustaining basal HIF-1 activity and promoting cancer progression.

Regulation of HIF-1 hydroxylation by multiple mechanisms.

HIF-1α hydroxylases are 2-OG utilizing dioxygenases that also require Fe(ll), molecular oxygen and ascorbate. The proposed sequential molecular mechanism for 2-OG dioxygenase activity first involves bidentate ligation of the apoenzyme Fe(ll) complex by the 1-carboxylate and 2-oxoacid functional groups of 2-OG. Binding of the 5-carboxylate to a distinct subsite, as well as binding of substrate, is also required for allosterically opening access of molecular oxygen to the axial coordination site of the ferrous iron. By assembling these components, the dioxygenases allow molecular oxygen to catalyze the insertion of one oxygen atom into the 2-carbon of 2-OG to form succinate and CO_2, with

the other oxygen atom likely forming a ferryl intermediate that subsequently produces substrate hydroxylation (reviewed in Hanauske-Abel and Popowicz, 2003; Schofield and Ratcliffe, 2004). Reduced availability of the co-substrates oxygen (by hypoxia), iron (by DFO), or 2-OG (by artificial 2-OG analogs such as NOG or DMOG) is a widely appreciated means of inhibiting these enzymes.

HPH inactivation may physiologically regulate HIF-1.

The 2-OG dependent dioxygenases can also be syn-catalytically inactivated. This means that as a result of catalyzing iron mediated oxidations, these enzymes either become oxidized at critical amino acid residues over time or the redox state of the iron becomes useless in carrying out sustained reaction cycles. This syn-catalytic inactivation can be blocked or reversed by ascorbate (Hanauske-Abel and Popowicz, 2003). The reversal of basal HIF-1 activation by ascorbate and Fe(II) in cancer cells suggests that HPH inactivation may be a prominent feature of many cancer cells (Knowles, et al., 2003). HPH inactivation through oxidation of cellular Fe(ll) to Fe(lll) may account for the ability of ROS and NO to promote HIF-1α buildup under physiological and pathological conditions (Chandel et al.,1998; Metzen, et al., 2003). For example, as a result of JunD mutation, some cancer cells generate excessive ROS and accumulate HIF-1α in an ascorbate and cysteine reversible manner (Gerald et al., 2004). A similar mechanism may underlie HIF-1 activation by ROS produced during cytokine signaling (BelAibah et al., 2004) or radiation therapy (Moeller et al, 2004). NO produces a prolonged inhibition of HPH activity, which persists even after NO is thoroughly washed away (Figure 5E; Metzen et al., 2003). Reversal

of NO mediated HPH inhibition by dithiothreitol has been interpreted as support for the formation of inhibitory nitrosothiols on the HPH protein (Metzen et al., 2003). However, our data with ascorbate and Fe(II) mediated reversal suggest that NO also oxidizes HPH associated iron (Figure 5). Like ascorbate, cysteine and histidine can also modulate iron redox status (Myllylä et al., 1978; Winkler et al., 1984) and the relative cellular levels of these amino acids or glutathione could thus play a significant role in determining the basal HIF-1α degradation rate of many cells.

HPH inactivation mediates HIF-1 regulation by anaerobic metabolites.

We have shown that, in culture, glucose-derived 2-oxoacids make a much greater contribution basal HIF-1α than serum derived factors. Although we could not detect significant ROS or NO production upon treatment of cells with glucose, pyruvate or oxaloacetate, we did find ascorbate, cysteine, histidine and Fe(II) to reverse HIF-1 activation by these stimuli. Pyruvate and oxaloacetate are not potent chelators or oxidants of free iron. However, these 2-oxoacids bound to the 2-OG site of HPHs and suspended HPH activity without suicidal inactivation. Selective reversal of this action by ascorbate, but not 2-OG, is consistent with unique but overlapping recognition sites for ascorbate and 2-OG on the dioxygenases (Majamaa, et al., 1986). Absence of a C5 carboxyl group may also allow pyruvate and oxaloacetate binding to interfere with allosteric enzyme mechanisms for the scheduled sequential binding of other reactants. This may allow oxidation of HPH-bound iron in the active site without catalyzing HIF-1α hydroxylation, thus explaining reactivation by ascorbate and Fe(II). Despite the

requirement of Fe(II) and ascorbate for observable activity of *in vitro* translated HPHs, we were still able to uncover inhibition of by pyruvate and oxaloacetate. Furthermore, the ability of these 2-oxoacids to induce HIF-1 regulated genes suggests that they may also regulate hydroxylation of Asn803 of HIF-1α by FIH-1.

2-oxoacids as metabolic signaling molecules.

The origin of pyruvate, oxaloacetate and the branched chain 2-oxoacids via diverse metabolic routes underscores the potential for HPH regulation by metabolic pathways. Despite a recent report suggesting a link between succinate buildup and HIF-1 activity (Selak et al 2005), we did not find a significant effect of succinate on HIF-1α accumulation, 2-OG binding to HPHs, or regulation ODD-GFP, or HRE-Luc expression. Although we ruled out differential cell permeability as a basis for this observation, we cannot rule out a cell-specific sensitivity to succinate or the inter-conversion of succinate into oxaloacetate in other studies (Selak et al 2005). In vivo*,* hypoxia is accompanied by a buildup of pyruvate, which is typically reflected as a rise in lactate. Dual regulation of HPHs by oxygen availability and 2-oxoacids may thus allow these enzymes to truly serve as sensors of anaerobiosis. Aerobic glycolysis is a hallmark of malignancy, wherein 2-oxoacids accumulate despite adequate O_2 availability (Warburg, 1930). In such a setting, the induction of HIF-1 by glycolytic metabolites, coupled in turn with the transcriptional upregulation of glycolytic enzymes and glucose transporters by HIF-1, could constitute an ominous feed-forward loop for malignant progression. Indeed, as with tumor hypoxia and HIF-1 expression, tumor lactate levels are

highly correlated with malignant progression, metastasis and poor clinical outcome (Walenta et al., 2004). The lowering of basal HIF-1α, basal expression of HIF-1 regulated genes, and basal invasiveness in cancer cells by ascorbate also supports previous observations of its anti-cancer effects (Cameron and Pauling, 1976). We suggest that the HPHs function as metabolic sensors in a broader context than previously appreciated and allow HIF-1 to induce adaptive genomic responses to changes in cellular oxygen levels, redox status and metabolism.

Experimental Procedures

Cell Culture and Hypoxia Treatment

Human U87, U251, U251-HRE (Rapisarda et al., 2002) and U373 glioma cells were cultured in Eagle's MEM medium (Mediatech). Hep3B Human hepatoma cells, 22B Human head and neck cancer cells, and human astrocytes (Clonetics, lot 1F1475) were cultured in high glucose DMEM (Gibco). DU145 human prostate cancer cells and O22 human head and neck cancer cells were cultured in RPMI 1640 medium (Sigma). C6 ODD-GFP rat glioma cells (D'Angelo et al., 2002) were cultured in high glucose DMEM with 1.5mg/ml G418. All culture media were supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. Rat neurons were cultured in Neurobasal Media with B27 supplement (Gibco). For cell hypoxia treatment, the culture dishes were sealed in a modular incubator chamber, flushed with gas containing 1% O_2, 5% CO_2, and

94% N_2 for 5 minutes, and incubated in this environment at 37˚C for indicated times.

Antibodies and Chemical Reagents

Protein extraction, western blot analysis, and immunocytochemistry was performed as described (Lu, et al., 2002; Dalgard, et al., 2004). Mouse monoclonal anti HIF-1α antibodies were 610958 (BD Biosciences), and NB100-123 (Novus). Mouse monoclonal anti-GFP antibody was 1814460 (Roche). Mouse monoclonal anti β-actin antibody was ab6276-100 (Abcam). Dimethyloxalylglycine was D1070 (Frontier Scientific). All other chemicals were from Sigma.

***In Vitro* Translation and HPH Prolyl Hydroxylation Assay**

Human HPH-1,2,3 and VHL pcDNA3.1/V5-HIS vectors (Bruick and McKnight, 2001) (kindly provided by R. Bruick) were used for TNT Coupled Reticulocyte Lysate *in vitro* transcription/translation (Promega). Cytoplasmic extract for HPH activity was made by lysing cell pellets in assay buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT) at 4˚C using a Dounce homogenizer, centrifuging at 20,000 g for 15 min and collecting supernatant for the assay. HPH enzymatic reactions were performed as described (Bruick and McKnight, 2001). For each reaction, either 7.5 μL of IVT products or 100 μg of cell extract protein were used as source of the enzyme. Activity was measured by scintillation counting or autoradiography after gel electrophoresis on a 10-20% Novex Tris Glycine gradient gel (Invitrogen).

2-Oxoglutarate:HPH Binding Assay

Epoxy-Activated Sepharose (Amerisham) coupled to 2-oxoglutarate was produced using the recommended manufacturer's protocol (Anzellotti, et al., 2000). 25,000 cpm of [^{35}S]-labeled human HPH protein was added to 100 µL of 2-oxoglutarate sepharose gel. Gel was washed four times with the indicated buffer. Analysis of HPH binding was measured by scintillation counting.

VHL:ODD Binding Assay

35,000 cpm of [^{35}S]-labeled human VHL protein was added to 1 µg of hydroxylated HIF peptide bound to Ultralink ImmunoPure Immoblized Streptavidin beads (Pierce) in the indicated buffer. The beads were washed three times with cold NTEN buffer and bound [^{35}S]-VHL was measured by scintillation counting.

HIF-1 Reporter Assays

HIF-1 luciferase reporter assay was performed using U-251-HRE cells as described (Rapisarda et al, 2002).

RT-PCR and Quantitative RT-PCR Analysis

Total RNA was isolated using the RNeasy kit (Qiagen). For RT-PCR, 1 µg of total RNA was used with the SUPERSCRIPT One-Step System (Invitrogen). VEGF, GLUT3, and β-Actin primers were described previously (Lu et al., 2002). For quantitative PCR analysis, the iQ SYBR Green Supermix and MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) was used. Total RNA (5µg) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). The following primers were used: for rat HO-1 (GenBank accession no. NM_012580), forward = 5'-AAGAGGCTAAGACCGCCTTC-3',

reverse = 5'-CCTCTGGCGAAGAAACTCTG-3'; for human CAIX (GenBank accession no. NM_001216), forward = 5'-CACTCCTGCCCTCTGACTTC-3', reverse = 5'-AGAGGGTGTGGAGCTGCTTA-3'; for human GLUT3 (GenBank accession no. NM_006931), forward = 5'-TGACGATACCGGAGCCAATG-3', reverse = 5'-TCAAAGGACTTGCCCAGTTT-3'; for human MMP-2 (GenBank accession no. NM_004530), forward = 5'-GTGGATGCCGCCTTTAACT-3', reverse = 5'-GGAAAGCCAGGATCCATTTT-3'. Primer temperature, concentration, and cDNA dilution optimizations were conducted before analysis and specific single-band amplification was verified through multicomponent analysis and sequencing of amplified products.

Cell Invasion Assay

Cell invasion experiments were performed using 24-well Biocoat Matrigel™ Invasion Chambers with an 8-μm pore polycarbonate filter according to manufacturer's instructions (#35-4480, Beckton Dickinson Labware). Cells in the growing phase were trypsinized and resuspended at a concentration of 1×10^5 cells/ml in media with 0.5% FBS. The lower compartment of the plates received 750 μl of serum free or serum media. All drug or serum treatments were added to the lower compartment of the plate prior to cell plating. 5×10^4 cells were plated in each insert and allowed to invade for 48 hours at 37°C in a humidified incubator with 21% O_2. Cells that remained inside the insert after 48 hours were thoroughly wiped with a cotton swab and invading cells were fixed and stained using Diff-Quick Stain Solution (Dade Begring). Stained invading cells were quantified by counting in 10 predetermined fields at 20x magnification.

Cellular reactive oxygen species and nitrite measurements

Cells (80% confluency) were loaded with 5 μM CM-H_2DCFDA (Molecular Probes) in Krebs buffer for 1h and then treated with 2 mM pyruvate or oxaloacetate for 2h. Positive controls were spiked twice with 40 μM H_2O_2. Cells were then collected, washed twice with PBS, and resuspended in PBS/0.1% (w/v) BSA before measuring fluorescence in a Coulter flow cytometer. Nitrite concentration in culture medium was measured as described (Misko et al., 1993).

VEGF ELISA, Glucose Metabolites, and Cellular ATP Assays

Culture medium VEGF was measured via ELISA (Quantikine). Glucose, lactate, and pyruvate were measured in phenol-red free DMEM using a CMA 600/ Microdialysis Analyzer (CMA Microdialysis AB). Cellular ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche).

Animal Experiments

Rat pups (14d old) were made hypoxic for 4h in a modulator incubator chamber, flushed continually with gas containing 8% O_2, 92% N_2. Pyruvate and oxaloacetate were injected intraperitoneally at a dose of 3g/kg in normal saline and animals were sacrificed after 4 hours.

Acknowledgements

This work was funded by NIH grant NS37814, and DOD grants MDA-905-02-2-0005 and MDA-905-03-2-0001 to A.V. C.L.D. and T.M. received fellowships from the Henry Jackson Foundation. The authors are grateful to H. Hanauske-Abel and R. Bruick for critical reading and valuable discussions of this work. R. Bruick,

S. McKnight, G. D'Angelo, and G. Melillo provided key reagents used in this work.

Figure legends.

Figure 1. 2-Oxoacid metabolites induce ascorbate-reversible HIF-1α. (A) Nuclear HIF-1α protein in normoxic human cancer cell lines grown ± ascorbate (Asc) for 24h in media containing 25mM (22B), 11.1mM (MCF-7, DU145) or 5.5mM glucose (U87, U251). U251 and U87 were also switched for 4h to Krebs buffer modified as indicated with specific metabolic fuels or intermediary metabolites. In the unlabeled lanes cells were treated only with glucose-free Krebs buffer. (B) Structures of key metabolic intermediates. Boxes indicate HIF-1α inducing natural metabolites (straight line) and artificial 2-OG analogs (dashed line). (C) Human neoplastic cells and normal astrocytes were cultured for 4h in glucose-free Krebs buffer supplemented with 3mM glucose, pyruvate or oxaloacetate. Rat brain nuclear extracts were analyzed 4h after hypoxia or 3g/Kg i.p. pyruvate or oxaloacetate injection. Primary rat cortical neurons were studied in neurobasal media following indicated treatments for 4h. Neuronal HO-1 gene expression was analyzed by normalized quantitative RT-PCR following 8h exposure to 1% O_2 or 1mM oxaloacetate. (D) The indicated cells were cultured in glucose-free Krebs buffer under hypoxia (1% O_2) or in normoxia with either DMOG (0.5mM), pyruvate (3mM), or oxaloacetate (3mM). Nuclear HIF-1α accumulation was measured at 4h and HRE luciferase activity was determined at 8h. Cultures also contained 2-OG (10mM) or ascorbate (100μM) where indicated.

Figure 2. Cysteine and histidine prevent metabolic HIF-1α accumulation. (A) Nuclear HIF-1α in U87 cells cultured for 4h in either fresh MEM or Krebs buffer. Amino acid (aa) and vitamin (Vit) additives found in MEM were added to Krebs buffer where indicated and pyruvate (1mM) was substituted for glucose where indicated. (B) Glucose-free Krebs was reconstituted with pyruvate (1mM) and individual amino acid at concentrations found normally in complete MEM. Nuclear HIF-1α was determined after 4h culture. Alternatively, each amino acid was individually raised to 5X its normal concentration in complete MEM and cells cultured for 2 days. (C) U87 cells were cultured for 4h in glucose-free Krebs buffer containing 150μM DFO, or 1mM pyruvate or oxaloacetate. Cells were additionally exposed to L- or D-isomers of cysteine and histidine (250μM), reduced glutathione (GSH) or 1% O_2 where indicated. One experiment with DU145 cells is also shown. (D) RT-PCR expression of VEGF, GLUT3, and actin in U87 cells following 2d culture in MEM containing 5X cysteine, histidine, or phenylalanine. Luciferase activity was determined in U251-HRE cells cultured in glucose-free Krebs containing either pyruvate or oxaloacetate (2mM) ± cysteine or histidine (250μM) for 8h.

Figure 3. Pyruvate and oxaloacetate block oxygen-dependent protein degradation. (A) Nuclear HIF-1α in U251 cells treated with indicated doses of H_2O_2 or DETA-NO for 4h. Media H_2O_2 and nitrite in cells were determined after 4h culture in glucose-free Krebs (control) supplemented with either 40μM H_2O_2, 3

mM pyruvate, 3mM oxaloacetate, or 300μM DETA-NO. (B) Nuclear HIF-1α levels in U87 grown for 4h in glucose-free media alone (lanes 1,7,11) or with added pyruvate or oxaloacetate in the presence of butyrate (Butyr, 10mM), trichostatin A (TSA, 300ng/ml), wortmannin (Wort, 1μM), LY294002 (LY, 20μM), rapamycin (Rapa, 100nM), or geldanamycin (Gelda, 3μM). (C) GFP expression in ODD-GFP transfected C6 glioma cells was measured by fluorescence microscopy or western blotting. Cells were grown for 24h in MEM or 4h in Krebs buffer modified to contain the indicated amounts of glucose, or 2mM oxaloacetate, succinate, or pyruvate. Ascorbate (100μM) and the amino acids histidine, cysteine and asparagine (0.5mM each) were added where indicated.

Figure 4. Pyruvate and oxaloacetate interact with and inhibit HPHs. (A) Binding of ^{35}S-pVHL to the hydroxylated HIF ODD peptide was measured in the presence or absence of 10mM pyruvate or oxaloacetate. (B) Binding of the ^{35}S-HPHs to 2-OG sepharose was measured in the presence or absence of 200μM Fe(II). (C) Binding of ^{35}S-HPH-1 to 2-OG was measured in the presence of free2-OG or succinate (10mM each). (D) The binding of ^{35}S-HPH-2 to 2-OG was measured in the presence of pyruvate or oxaloacetate (10mM). HPH activity was determined in the presence of increasing doses of (E) 2-OG, Fe(II) and ascorbate (Asc) while the other components were kept constant (2mM 2-OG, 250μM $FeSO_4$, 2mM ascorbate, 1mM DTT, streptavidin beads pre-incubated with 1μg biotinylated HIF-1α peptide, 20ng IVT product) or (F) cysteine and histidine, in the absence of ascorbate and DTT. (G) Pyruvate and oxaloacetate (2mM) could not replace 2-

OG as productive substrates. (H) Pyruvate and oxaloacetate reduced enzymatic activity in a specific window of ascorbate concentrations. Quantitative data are means +/- SEM. (* $P<0.05$, ** $P<0.01$).

Figure 5. Reversible inactivation of cellular HIF-1 hydroxylation by 2-oxoacids. (A) U251 cultured for 4h in glucose-free Krebs either in 1%O_2 or with 3mM pyruvate in 21% O_2 were washed 3x and maintained in oxygenated glucose-free Krebs buffer until formalin fixation and staining for HIF-1α immunoreactivity at the indicated times. (B) U87 were treated in glucose-free Krebs buffer ± 1mM pyruvate or oxaloacetate. After 4h, cells were washed in glucose-free Krebs for various times ± 100μM ascorbate, 5mM GSH or the MEM amino acid mixture being included in the wash. (C) U251 were cultured for 4h in glucose-free Krebs buffer ± the indicated additions of 2mM pyruvate, 2mM oxaloacetate, or 1mM DMOG or 100μM ascorbate. After washing, whole cell extracts were prepared and used as the source of HPH enzyme in ^{35}S-pVHL pull down assays with no exogenous addition of iron, or ascorbate. (D) Experiments similar to (C) were performed except ascorbate or $FeSO_4$ were added to the extract at 100μM each. (E) Nuclear HIF-1 accumulation was monitored in U251 grown for 4h in glucose-free Krebs buffer supplemented with either DETA-NO (300μM), pyruvate (3mM) or oxaloacetate (3mM). Cells were co-incubated with ascorbate (100μM), Fe(II) (100μM) or GSH (5mM) as indicated. Decay of NO-induced HIF-1α was followed after washing with glucose-free Krebs ± Fe(II) or Fe(III) supplementation (100μM each). (F) Decay of HIF-1α induced by either pyruvate, oxaloacetate, or DFO

was followed after washing with glucose-free Krebs ± Fe(II) or Fe(III) supplementation (100μM each). Unlabeled lanes indicate cells cultured in glucose-free Krebs buffer alone.

Figure 6. Basal HIF-1 activation through glucose metabolism correlates with invasive cancer phenotype. HNSSCA cell lines O22 and 22B display differential basal levels of HIF-1α (A), VEGF (B), invasiveness (C), glucose consumption (D), pyruvate production (E), and lactate accumulation (F). G. Nuclear HIF-1α levels were monitored in 22B cells cultured in complete DMEM or glucose-free medium ± serum. H. Hypoxic responsiveness of 22B cultured as in (G). I. Nuclear HIF-1α levels were monitored in 22B cultured in complete DMEM with added pyruvate, Fe(II), or Fe(III). (J-K) Selective ascorbate reversal of basal HIF-1α in 22B. (L) Effect of ascorbate on basal expression of HIF-1 regulated genes in 22B cells. (M) Effect of ascorbate on basal invasiveness of 22B cells.

Supplemental Figure S1. (A) ATP levels were measured in U251 cells following culture under the indicated conditions. (B) U251 cells were permeabilized with 10μM digitonin and cultured in glucose-free Krebs buffer under hypoxia (1%O_2), or normoxia (21% O_2) with pyruvate, oxaloacetate, succinate, ATP, or AMP (2mM each). Nuclear HIF-1α was measured following 4h treatment. ODD-GFP C6 were also permeabilized with 10μM digitonin and cultured for 4h in glucose-free Krebs containing the indicated additions. GFP was measured in whole cells extracts. (C-D) Raw data from ^{35}S-pVHL pull down assays for HIF-1α

hydroxylation showing effect of varying ascorbate +/- 1mM pyruvate or oxaloacetate.

References:

Anzellotti, D., and Ibrahim, R.K. (2000). Novel flavonol 2-oxoglutarate dependent dioxygenase: affinity purification, characterization, and kinetic properties. Arch. Biochem. Biophys. *382*, 161-172.

BelAiba, R.S., Djordjevic, T., Bonello, S., Flugel, D., Hess, J., Kietzmann, T., Gorlach, A. (2004). Redox-sensitive regulation of the HIF pathway under non-hypoxic conditions in pulmonary artery smooth muscle cells. Biol. Chem. *385*, 249-57.

Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337–1340.

Cameron E, and Pauling L. (1976). Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. Proc. Natl. Acad. Sic. USA *73*, 3685-3689.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C., and Schumacker, P.T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. USA *95*, 11715–11720.

Cohen, N.A., Lai, S.Y., Zoiber A.F., and Zoiber, B.L. (2004). Dysregulation of hypoxia inducible factor-1α in head and neck cell lines correlates with invasive potential. Laryngoscope *114*, 418-423.

Dalgard C., Lu, H., Mohyeldin, A., and Verma (2004). Endogenous 2-oxoacids differentially the expression of oxygen sensors. Biochem. J. *380*,419-424.

D'Angelo G., Duplan E., Vigne P., Frelin C. (2002). Cyclosporin A prevents the hypoxic adaptation by activating hypoxia-inducible factor-1alpha Pro-564 hydroxylation. J. Biol. Chem. *271*, 5406-11.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., et al. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43–54.

Gerald, D., Berra, E., Frapart, Y.M., Chan, D.A., Giaccia, A.J., Mansuy, D., Pouyssegur, J., Yaniv, M., and Mechta-Grigoriou, F. (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell *118*, 781-794.

Hanauske-Abel, H.M. and Popowicz, A.M. (2003) The HAG mechanism: a molecular rationale for the therapeutic application of iron chelators in human diseases involving the 2-oxoacid utilizing dioxygenases.
Curr. Med. Chem. *10*, 1005-19.

Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., et al. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J. Biol. Chem. 277, 26351-26355.

Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA *95*, 7987-7992.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*, 464–468.

Jaakkola, P., Mole, R.D., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Von Kriegsheim, A., Hebenstreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF-1 to the von Hippel-Lindau ubiquitylation complex by O2-

regulated prolyl hydroxylation. Science *292*, 468–472.

Kaule G, Timpl R, Gaill F, Gunzler V. (1998) Prolyl hydroxylase activity in tissue homogenates of annelids from deep sea hydrothermal vents. Matrix Biol. *17*, 205-12.

Knowles, H.J., Raval, R.R., Harris, A.L., and Ratcliffe, P.J. (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res. *63*, 1764-1768.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A, Whitelaw, M.L.., and Bruick, RK. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. *16*, 1466-1471.

Lu, H., Forbes, R. A., and Verma, A. (2002). Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem. 277, 23111-23115.

Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. *15*, 2675–2686.

Majamaa, K., Turpeenniemi-Hujanen, T.M., Latipää, P., Günzler, V., Hanauske-Abel, H.M., Hassinen, E., and Kivirikko, K.I. (1985). Differences between collagen hydroxylases and 2-oxoglutarate dehydrogenase in their inhibition by structural analogs of 2-oxoglutarate. Biochem. J. *229*, 127-133.

Majamaa, K., Günzler, V., Hanauske-Abel, H.M., Myllylä, R., Kivirikko, K.I. (1986). Partial identity of the 2-oxoglutarate and ascorbate binding sites of prolyl 4-hydroxylase. J. Biol. Chem. *261*, 7819 – 7823

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J., and Brüne, B. (2003). Mol. Cell. Biol. *14*, 3470-3481.

Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. (1993). A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. *214*, 11-16.

Moeller, B. J., Cao, Y., Li, C. Y., and Dewhirst, M. W. (2004). Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free

radicals, and stress granules. Cancer Cell *5*, 429-441.

Myllylä, R., Kuutti-Savolainen, E.R., and Kivirikko, K.I. (1978) The role of ascorbate in the prolyl hydroxylase reaction. Biochem. Biophys. Res. Commun. *83*, 441-448.

Rapisarda, A., Uranchimeg, B., Scudiero, D. A., Selby, M., Sausville, E. A., Shoemaker, R. H., and Melillo, G. (2002). Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res. *62*, 4316-4324.

Salceda, S., and Caro, J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J. Biol. Chem. *272*, 22642-22647.

Selak, M.A., Armour, S.M., Mackenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B., Gottlieb, E. (2005). Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell. *7*, 77-85.

Semenza, G.L. (2003). Targeting HIf-1 for cancer therapy. Nat. Rev. Cancer *3*, 721-732.

Schofield, C.J. and Ratcliffe P.J. (2004). Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell. Biol. *5*, 343-354.

Walenta S, Schroeder T, Mueller-Klieser W. (2004) Lactate in solid malignant tumors: potential basis of a metabolic classification in clinical oncology. Curr. Med. Chem. *16*, 2195-204.

Warburg, O. The metabolism of tumors. London: Constable Press, 1930.

Winkler, P., Schaur, R.J., Schauenstein, E. (1984) Selective promotion of ferrous ion-dependent lipid peroxidation in Ehrlich ascites tumor cells by histidine as compared with other amino acids. Biochim. Biophys. Acta.*796*, 226-31.

Zhang, X., Huang, C. J., Nazarian, R., Ritchie, T., de Vellis, J. S., and Noble, E. P. (1997). Isolation of nuclear proteins from human brains. Biotechniques *22*, 848-850.

Zhong, H., Hanrahan, C., van der Poel, H., Simons, J.W. (2001). Hypoxia-inducible factor 1alpha and 1beta proteins share common signaling pathways in human prostate cancer cells. Biochem. Biophys. Res. Commun., *284*, 352-356.

Figure 1.

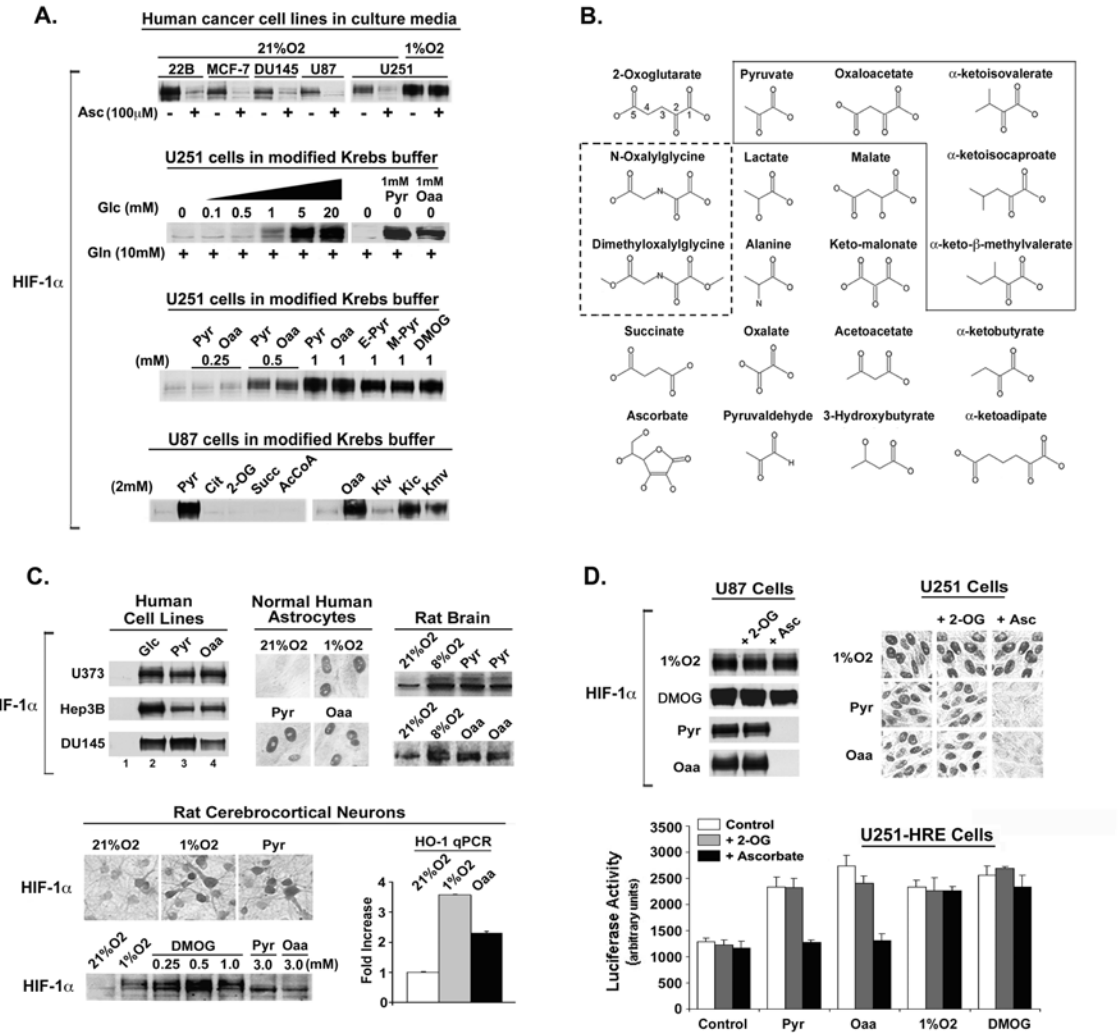

A.
Human cancer cell lines in culture media
21%O2
1%O2
22B
MCF-7
DU145
U87
U251
Asc (100μM)
U251 cells in modified Krebs buffer
Glc (mM)
0 0.1 0.5 1 5 20 0
1mM Pyr
1mM Oaa
Gln (10mM)
HIF-1α
U251 cells in modified Krebs buffer
Pyr Oaa Pyr Oaa Pyr Oaa E-Pyr M-Pyr DMOG
(mM)
0.25 0.5 1 1 1 1 1
U87 cells in modified Krebs buffer
(2mM)
Pyr Cit 2-OG Succ AcCoA Oaa Kiv Kic Kmv
B.
2-Oxoglutarate
Pyruvate
Oxaloacetate
α-ketoisovalerate
N-Oxalylglycine
Lactate
Malate
α-ketoisocaproate
Dimethyloxalylglycine
Alanine
Keto-malonate
α-keto-β-methylvalerate
Succinate
Oxalate
Acetoacetate
α-ketobutyrate
Ascorbate
Pyruvaldehyde
3-Hydroxybutyrate
α-ketoadipate
C.
Human Cell Lines
Glc Pyr Oaa
U373
Hep3B
DU145
HIF-1α
1 2 3 4
Normal Human Astrocytes
21%O2 1%O2
Pyr Oaa
Rat Brain
21%O2 8%O2 Pyr Pyr
21%O2 8%O2 Oaa Oaa
Rat Cerebrocortical Neurons
21%O2 1%O2 Pyr
HIF-1α
21%O2 1%O2 DMOG 0.25 0.5 1.0 Pyr 3.0 Oaa 3.0 (mM)
HIF-1α
HO-1 qPCR
21%O2 1%O2 Oaa
Fold Increase
0 1 2 3 4
D.
U87 Cells
+ 2-OG + Asc
1%O2
DMOG
Pyr
Oaa
HIF-1α
U251 Cells
+ 2-OG + Asc
1%O2
Pyr
Oaa
U251-HRE Cells
Control
+ 2-OG
+ Ascorbate
Luciferase Activity (arbitrary units)
0 500 1000 1500 2000 2500 3000 3500
Control Pyr Oaa 1%O2 DMOG

Figure 2.

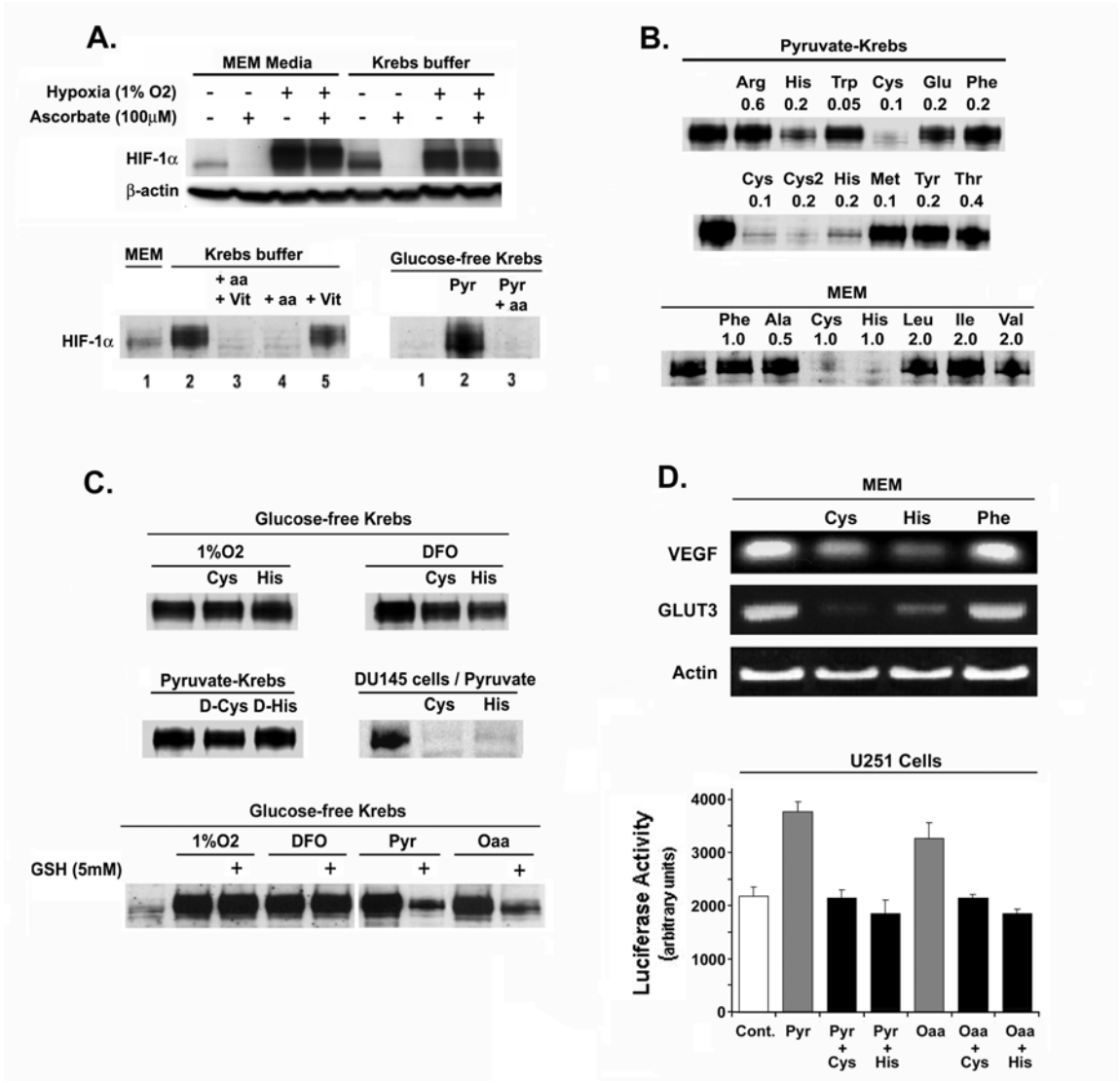

A.
MEM Media
Krebs buffer
Hypoxia (1% O2)
Ascorbate (100μM)
HIF-1α
β-actin
MEM
Krebs buffer
+ aa
+ Vit
+ aa
+ Vit
Glucose-free Krebs
Pyr
Pyr + aa
HIF-1α
1 2 3 4 5
1 2 3
B.
Pyruvate-Krebs
Arg 0.6
His 0.2
Trp 0.05
Cys 0.1
Glu 0.2
Phe 0.2
Cys 0.1
Cys2 0.2
His 0.2
Met 0.1
Tyr 0.2
Thr 0.4
MEM
Phe 1.0
Ala 0.5
Cys 1.0
His 1.0
Leu 2.0
Ile 2.0
Val 2.0
C.
Glucose-free Krebs
1%O2
Cys
His
DFO
Cys
His
Pyruvate-Krebs
D-Cys
D-His
DU145 cells / Pyruvate
Cys
His
Glucose-free Krebs
1%O2
DFO
Pyr
Oaa
GSH (5mM)
+
+
+
+
D.
MEM
Cys
His
Phe
VEGF
GLUT3
Actin
U251 Cells
Luciferase Activity
(arbitrary units)
4000
3000
2000
1000
0
Cont.
Pyr
Pyr + Cys
Pyr + His
Oaa
Oaa + Cys
Oaa + His

Figure 3.

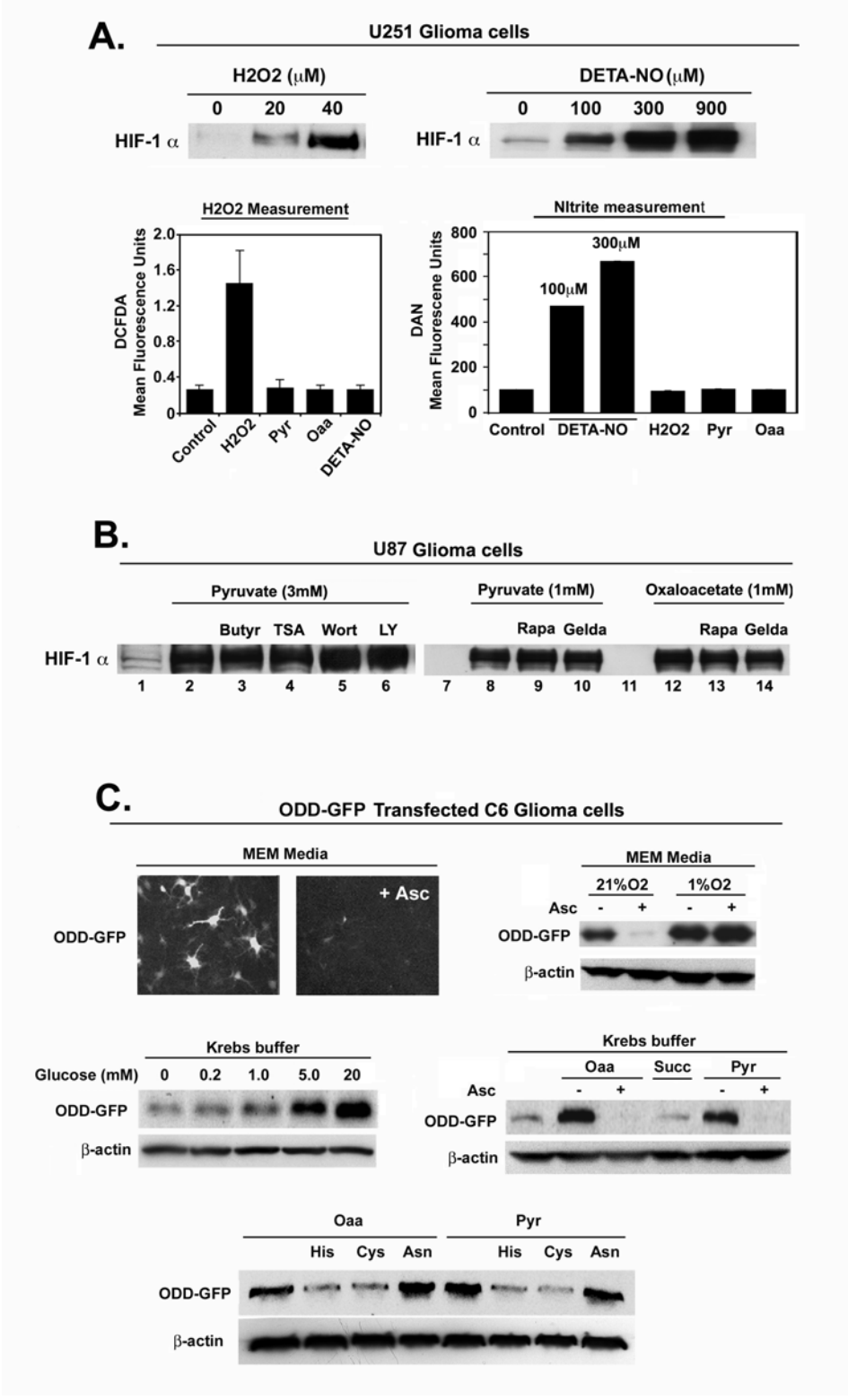

A.
U251 Glioma cells
H2O2 (μM)
0 20 40
HIF-1 α
DETA-NO (μM)
0 100 300 900
HIF-1 α
H2O2 Measurement
DCFDA Mean Fluorescence Units
2.0
1.6
1.2
0.8
0.4
0
Control
H2O2
Pyr
Oaa
DETA-NO
Nitrite measurement
DAN Mean Fluorescene Units
800
600
400
200
0
100μM
300μM
Control
DETA-NO
H2O2
Pyr
Oaa
B.
U87 Glioma cells
Pyruvate (3mM)
Butyr TSA Wort LY
Pyruvate (1mM)
Rapa Gelda
Oxaloacetate (1mM)
Rapa Gelda
HIF-1 α
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C.
ODD-GFP Transfected C6 Glioma cells
MEM Media
ODD-GFP
+ Asc
MEM Media
21%O2
1%O2
Asc - + - +
ODD-GFP
β-actin
Krebs buffer
Glucose (mM) 0 0.2 1.0 5.0 20
ODD-GFP
β-actin
Krebs buffer
Oaa
Succ
Pyr
Asc - + - +
ODD-GFP
β-actin
Oaa
His Cys Asn
Pyr
His Cys Asn
ODD-GFP
β-actin

Figure 4.

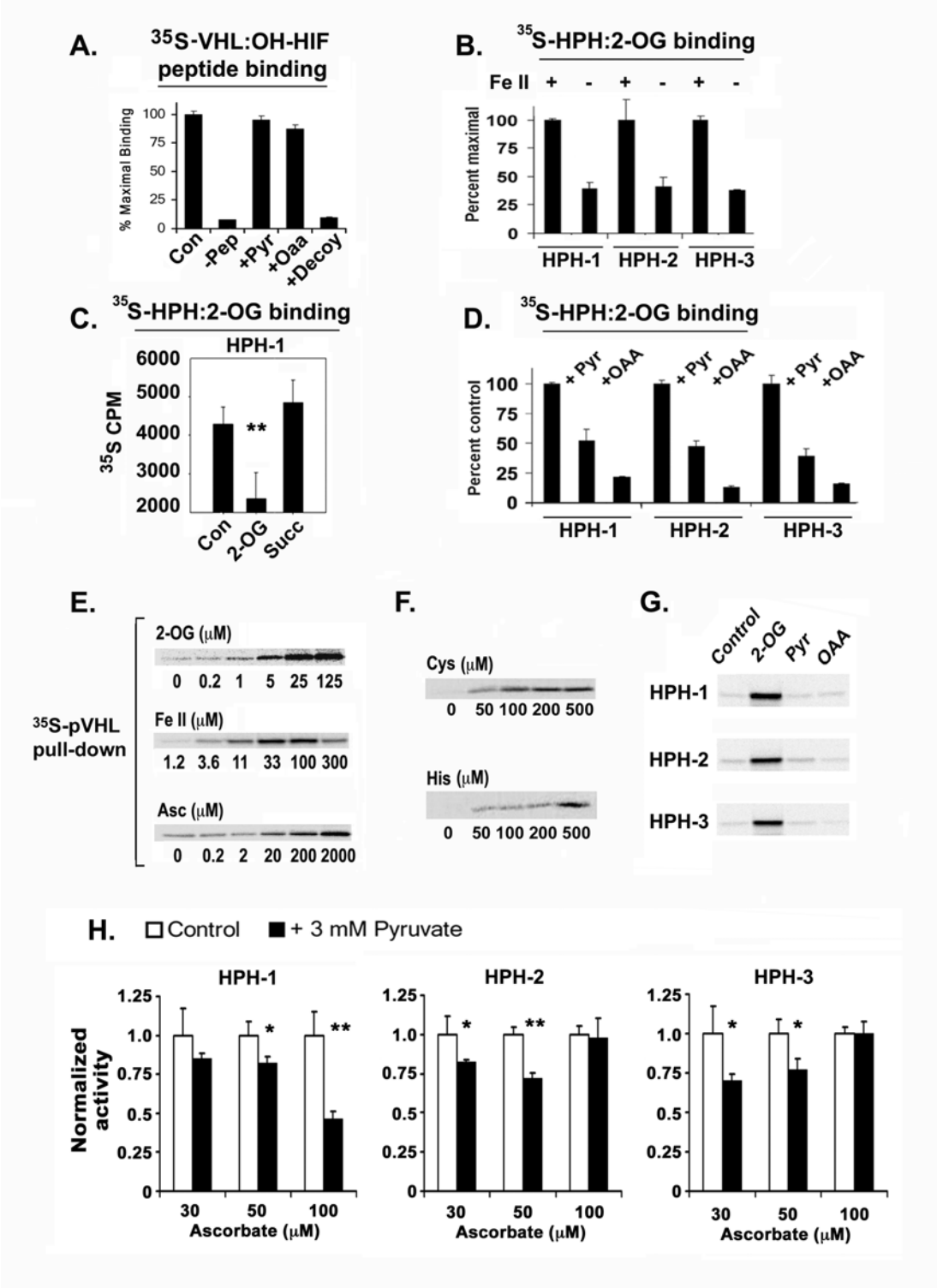

A.
35S-VHL:OH-HIF peptide binding
% Maximal Binding
100
75
50
25
0
Con
-Pep
+Pyr
+Oaa
+Decoy
B.
35S-HPH:2-OG binding
Fe II + - + - + -
Percent maximal
100
75
50
25
0
HPH-1
HPH-2
HPH-3
C.
35S-HPH:2-OG binding
HPH-1
35S CPM
6000
5000
4000
3000
2000
**
Con
2-OG
Succ
D.
35S-HPH:2-OG binding
Percent control
100
75
50
25
0
+ Pyr
+OAA
HPH-1
HPH-2
HPH-3
E.
35S-pVHL pull-down
2-OG (μM)
0 0.2 1 5 25 125
Fe II (μM)
1.2 3.6 11 33 100 300
Asc (μM)
0 0.2 2 20 200 2000
F.
Cys (μM)
0 50 100 200 500
His (μM)
0 50 100 200 500
G.
Control
2-OG
Pyr
OAA
HPH-1
HPH-2
HPH-3
H.
Control
+ 3 mM Pyruvate
HPH-1
HPH-2
HPH-3
Normalized activity
1.25
1.0
0.75
0.5
0.25
0
*
**
30
50
100
Ascorbate (μM)

Figure 5.

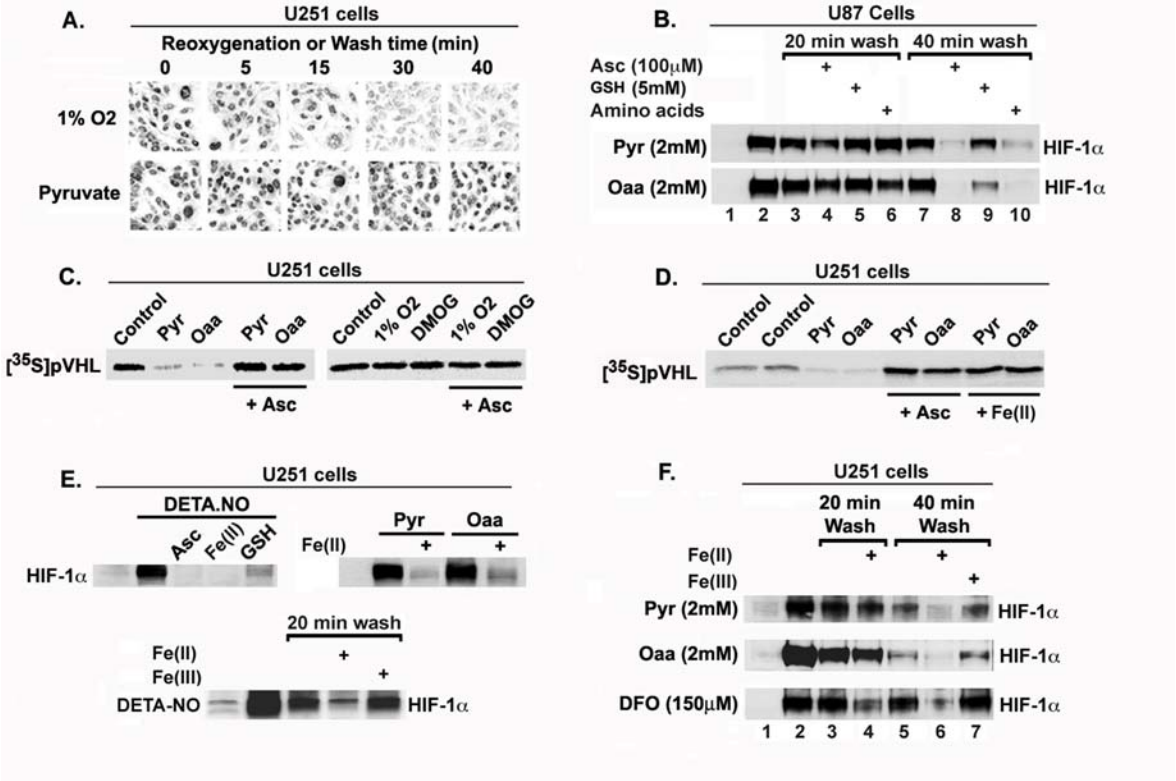
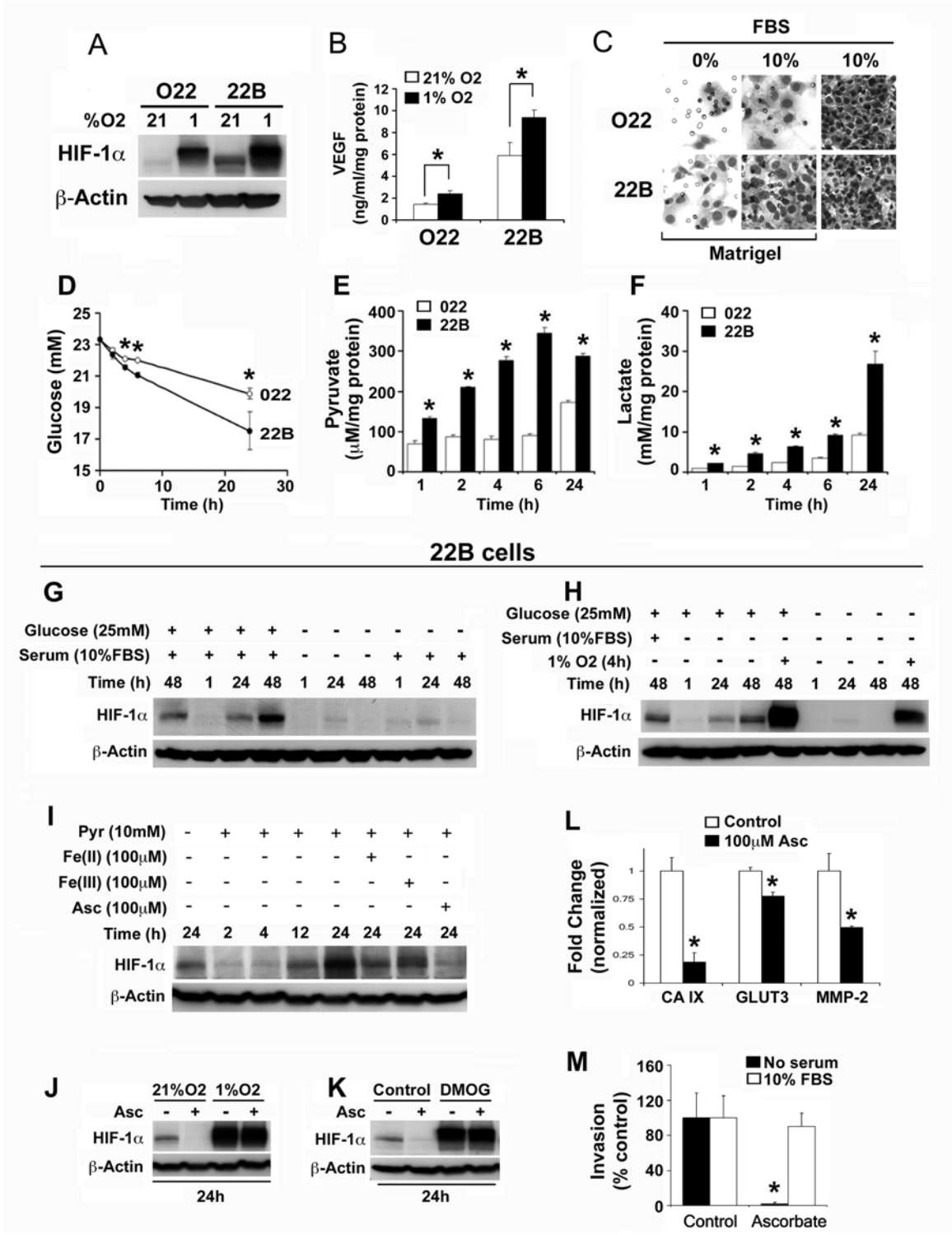
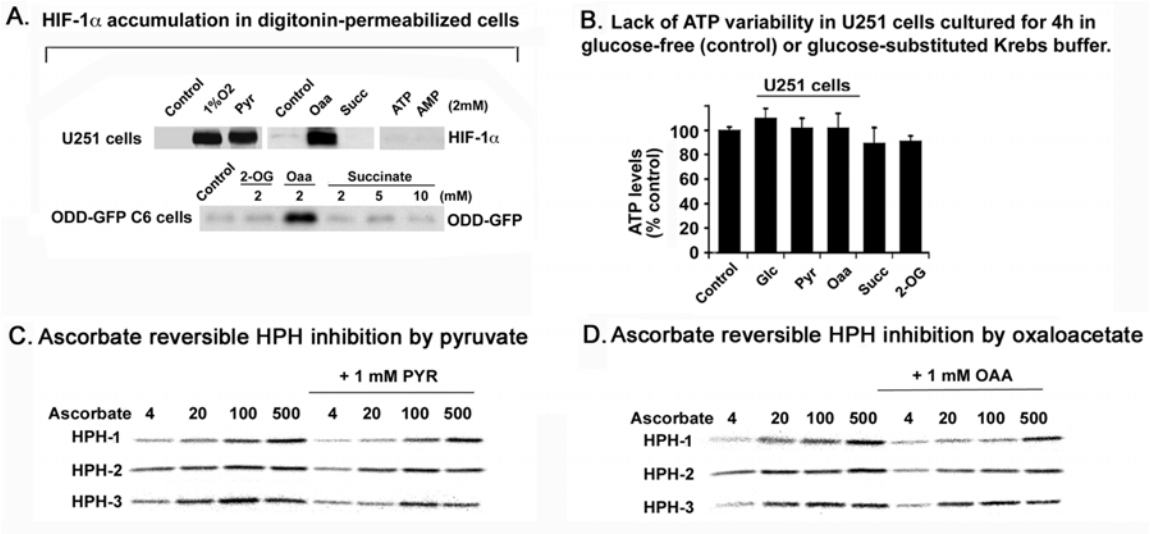

A.
U251 cells
Reoxygenation or Wash time (min)
0
5
15
30
40
1% O2
Pyruvate
B.
U87 Cells
20 min wash
40 min wash
Asc (100μM)
GSH (5mM)
Amino acids
Pyr (2mM)
Oaa (2mM)
HIF-1α
1 2 3 4 5 6 7 8 9 10
C.
U251 cells
Control
Pyr
Oaa
Pyr
Oaa
Control
1% O2
DMOG
1% O2
DMOG
[35S]pVHL
+ Asc
+ Asc
D.
U251 cells
Control
Control
Pyr
Oaa
Pyr
Oaa
Pyr
Oaa
[35S]pVHL
+ Asc
+ Fe(II)
E.
U251 cells
DETA.NO
Asc
Fe(II)
GSH
HIF-1α
Fe(II)
Pyr
Oaa
20 min wash
Fe(II)
Fe(III)
DETA-NO
HIF-1α
F.
U251 cells
20 min Wash
40 min Wash
Fe(II)
Fe(III)
Pyr (2mM)
Oaa (2mM)
DFO (150μM)
HIF-1α
1 2 3 4 5 6 7

Figure 6.


A
O22
22B
%O2
21
1
21
1
HIF-1α
β-Actin
B
21% O2
1% O2
VEGF (ng/ml/mg protein)
O22
22B
C
FBS
0%
10%
10%
O22
22B
Matrigel
D
Glucose (mM)
Time (h)
022
22B
E
Pyruvate (μM/mg protein)
Time (h)
F
Lactate (mM/mg protein)
Time (h)
22B cells
G
Glucose (25mM)
Serum (10%FBS)
Time (h)
HIF-1α
β-Actin
H
Glucose (25mM)
Serum (10%FBS)
1% O2 (4h)
Time (h)
HIF-1α
β-Actin
I
Pyr (10mM)
Fe(II) (100μM)
Fe(III) (100μM)
Asc (100μM)
Time (h)
HIF-1α
β-Actin
L
Control
100μM Asc
Fold Change (normalized)
CA IX
GLUT3
MMP-2
J
21%O2
1%O2
Asc
HIF-1α
β-Actin
24h
K
Control
DMOG
Asc
HIF-1α
β-Actin
24h
M
No serum
10% FBS
Invasion (% control)
Control
Ascorbate

Supplementary Figure S1.



SUMMARY OF PAPER 3

Rationale: HPH homologue expression patterns are known to be cell and tissue specific. Brain regions possess well-known differential vulnerability to hypoxia. Differential expression and regulation of the HPH oxygen sensors in the brain may provide valuable insight into brain injury mechanisms.

Objectives: In this paper, we set out to (a) determine the mRNA and protein expression patterns of HPH homologues in the rat brain and rat brain derived cells; (b) determine the regulation of HPH homologue expression by hypoxia in rat brain and rat brain derived cells; (c) determine the total HIF hydroxylation capacity of various brain regions isolated from normoxic and hypoxic rats.

Methods: These studies were performed in Wistar Hanover male rats and cultured rat C6 gliomas, rat embryonic cortical astrocytes and cortical neurons. Treated animals were exposed to 8% O_2 or 0.1% CO, while treated cells were exposed to 1% O_2 atmospheres. We examined HPH mRNA expression in rat cells and tissues by quantitative PCR analysis and in situ hybridization. HPH-1 protein expression was determined by western blotting. Total HPH hydroxylation capacity in rat brain regions was examined using the *in vitro* VHL pulldown HPH hydroxylation assay.

Findings: In this paper, we found that rat brain regions possess differential HPH mRNA expression and hydroxylation capacities. The regulation of HPH-1 and HPH-2 mRNA expression by hypoxia was also found to be differential in the rat brain regions examined. In comparative analysis between rat primary cortical astrocytes and neurons, HPH-3 was found to be significantly higher in cortical astrocytes. A similar analysis between rat

primary cortical astrocytes and C6 gliomas demonstrated higher HPH-1 expression in the primary cortical astrocytes.

Significance: The significant differences found in HPH expression and hydroxylation capacity between different rat brain regions and cell types defines unique capabilities of brain tissue to regulate regional responses to changing oxygen levels. Moreover, our findings suggest that the ability of brain tissue to adapt to changes in oxygen levels is diminished in an age related manner. These findings may provide insight into regional brain vulnerabilities to hypoxia, ischemia, and aging.

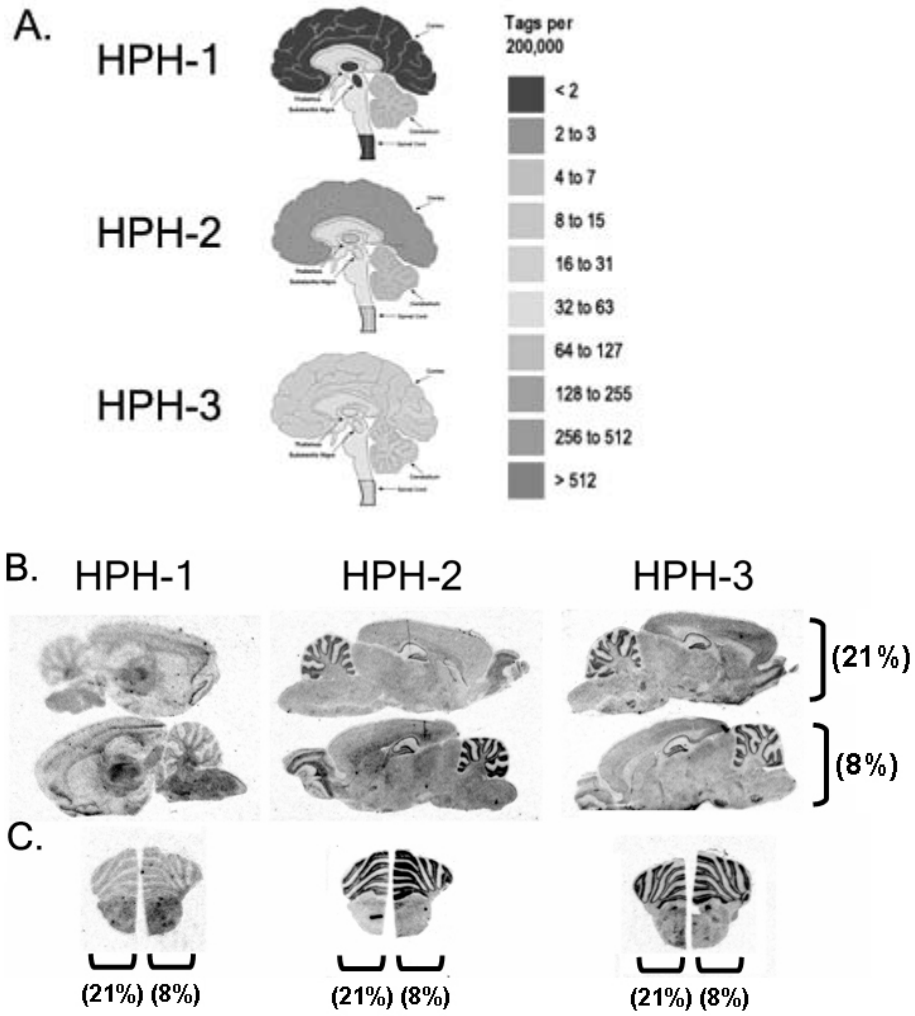


Figure 2. A. Schematic graphical data of human brain HPH gene expression from the SAGE (Serial Analysis of Gene Expression) database demonstrating potential differences in rat brain regions. B. HPH *in situ* hybridization of sagittal sections obtained from four week old male Wistar Hanover rats. Animals were either untreated (above) or treated with 8% O_2 for 8 hours (below) before sacrifice. C. HPH *in situ* hybridization of coronal sections obtained from four week old male Wistar Hanover rats. Animals were either untreated (left) or treated with 8% O_2 for 8 hours (right) before sacrifice. *In situ* hybridization expression mapping demonstrates differential regional HPH homologue expression as well as differential hypoxic induction of HPH homologue expression.

(In preparation)

DIFFERENTIAL REGIONAL EXPRESSION OF THE HIF PROLYL HYDROXYLASE MAMMALIAN OXYGEN SENSORS IN THE RAT BRAIN.

Clifton Lee Dalgard, Huasheng Lu, Guoqiang Xing, and Ajay Verma

Department of Neurology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD, 20814, Tel: 301-295-3840, Fax: 301-295-3825, averma@usuhs.mil

* To whom all correspondence should be addressed.

Supported by PHS Grant NS37814, and DOD grants (DBSCIP and USMCI) to A.V.

ABSTRACT

Cells have the capacity to respond to reduced oxygen tension by regulating the expression of genes involved in increasing oxygen supply and decreasing oxygen demand. The transcription factor HIF-1 (hypoxia inducible factor-1) is a central component to hypoxic adaptation and it is now well understood that under normal oxygen tensions, a trio of HIF prolyl hydroxylases (HPHs) hydroxylate two specific proline residues within a degradation domain of the alpha subunit of HIF-1. This hydroxyproline modification leads to ubiquitin-mediated proteasomal degradation of HIF-1α. HPH-mediated hydroxylation requires molecular oxygen. Thus, during hypoxia, HPH enzymatic activity is reduced, allowing HIF-1α to accumulate and upregulate gene expression. Mammalian HPH homologues have tissue specific expression patterns. Furthermore, all three mammalian HPH homologues are found in the rat genome. This paper investigates the specific expression patterns and enzymatic capacities of the three HPH homologues in rat brain tissue. We demonstrate differential HPH hydroxylation capacity as well as differential expression patterns of the HPH mRNA and protein in rat brain regions. Additionally, HPH expression profiles differed during comparative analysis of two rat brain cell types and a permanent rat glioma cell line. We propose that differential HPH profiles in rat brain tissues and cells may play a role in brain adaptations to hypoxic conditions found in stroke, cancer, and other oxygen-related neurological diseases.

INTRODUCTION

Cells and organisms require molecular oxygen to function, survive, and grow appropriately. During periods of acute and chronic hypoxia, cells adapt to these physiological conditions by regulating a complex set of genes involved in increasing oxygen supply and decreasing oxygen demand. These genes are important in several cellular and system processes including angiogenesis, hematopoeisis, energy metabolism, and cell survival (Metzen and Ratcliffe 2004). The central transcription factor responsible for the coordination of these events is the hypoxia inducible factor-1 (HIF-1). HIF exists as a heterodimer of an oxygen-regulated alpha subunit and a stable beta subunit (also known as the arylhydrocarbon receptor nuclear translocator, ARNT) (Wang, Jiang et al. 1995). HIF-1α and HIF-1β are both constantly expressed. However, under normoxic conditions HIF-1α is also constantly degraded. It is only during conditions of hypoxia which HIF-1α accumulates, translocates to the nucleus, heterodimerizes with HIF-1β, and binds to DNA promoters to activate target gene transcription. HIF has been characterized to be specifically responsible for the upregulation of over 40 genes (Schofield and Ratcliffe 2004), and potentially may be responsible for more with several hundred genes shown to be regulated by hypoxia (Koong, Denko et al. 2000; Wykoff, Pugh et al. 2000; Jiang, Zhang et al. 2003).

The manner by which reduction of cellular oxygen tension leads to the accumulation of HIF-1α is now well understood. HIF-1α possesses an oxygen-degradation domain (ODD) where two critical proline residues (Pro-402 and Pro-564) become hydroxylated under normoxic oxygen conditions ((Yu, White et al. 2001). These hydroxyproline motifs in this ODD domain are recognized and bound by the tumor

suppressor pVHL (von-Hippel-Lindau protein), a E3 ligase which polyubiquitinates HIF-1α and targets it for proteasomal degradation (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001). The class of enzymes which accomplish the hydroxylation of proline residues is the 2-oxoglutarate-dependent-oxygenase family (Schofield and Ratcliffe 2004). These enzymes were previously identified by their ability to hydroxylate proline residues in collagen, and studies have demonstrated they are dependent on ferrous iron to coordinate bonds with the substrates 2-oxoglutarate and O_2. This complex of Fe^{2+} / 2-oxoglutarate / O_2 leads to an oxidative decarboxylation reaction where one atom of oxygen is incorporated into 2-oxoglutarate (producing succinate and CO_2), while the second atom of oxygen is incorporated into the proline residue (producing hydroxyproline).

Three separate enzymes that hydroxylate HIF-1α have been identified and given various nomenclatures from the groups which first identified them (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001). HIF prolyl hydroxlases (HPH) is the nomenclature we use in this study. HPH-1 is also called PHD3 and EGLN3 (Figure 1). HPH-2 is identical to PHD2 and EGLN1. HPH-3 is also called PHD1 and EGLN2. Initial studies during the identification and enzymatic characterization of these enzymes were conducted mostly in permanent cell lines and only a few studies have been conducted to explore the tissue specific HPH expression *in vivo*. One study demonstrated overlapping and specific patterns of expression in mouse tissues for the HPH homologues. HPH-1 was found to be highly expressed in the heart and liver and possess lower expression levels in the brain. HPH-2 was moderately expressed in several tissues, while HPH-3 was found to be highly expressed in the testis and was the only homologue observed in that organ. These results are significant as differential tissue expression patterns may

provide insight into differential hypoxia sensitivity or different functions of the HPH homologues *in vivo*.

The brain is the most susceptible organ to decreased oxygen supply. Additionally, neurons are considered to be the most oxygen sensitive of all vertebrate cells. Thus, cellular responses to oxygen deprivation can demonstrate differential sensitivity and injury. For example, one study demonstrated that after hypoxic/ischemic injury, neuronal loss is most prominent in the hippocampus, globus pallidus, cerebellum, and inferior olives (Dijkhuizen, Knollema et al. 1998). In the same study, neuronal injury was significantly less in the substania nigra and diencephalic thalamus. The underlying reason for the differential sensitivity of these cells and regions in the mammalian brain remains entirely unknown. Also, the characterization of the HIF pathway in these regions is not well established.

In this paper, we investigated the properties of the HPH homologues in rat brain tissue and rat brain-derived cells. We determined the HPH enzymatic capacities of several rat brain regions in normoxic and hypoxia-exposed conditions. We also determined the specific HPH expression patterns in the rat brain utilizing qPCR and in situ hybridization. Differences in HPH hydroxylation capacity as well as differences in the expression patterns of the HPH mRNA and protein were found when comparing several rat brain regions. Additionally, HPH expression profiles differed during comparative analysis of two rat brain cell types and a permanent rat glioma cell line. Our data of differential HPH profiles in rat brain tissues and cells may provide insight into cellular adaptations and survival in brain tissue during hypoxic conditions that are commonly found in stroke, cancer, and other oxygen-related neurological diseases.

MATERIALS AND METHODS

Animal Treatments

For hypoxia treatments, Male Wistar Hanover rats of either 4 or 8 week old age were untreated or place into a modular incubator chamber and flushed continually with gas containing 8% O_2 and balance N_2 for 8 hours duration. Animals were then rapidly removed from the chamber and decapitated. Brains were quickly removed, frozen on aluminum foil placed on powered dry ice (-30 degrees Celsius), and stored at -70 degrees Celsius.

Cell Culture and Hypoxia Treatment

E17 rat primary cortical astrocytes and C6 gliomas were cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. E17 rat primary cortical neurons were cultured in Neurobasal Media with B27 supplement (Gibco). For cell hypoxia treatments, the culture dishes were sealed in a modular incubator chamber, flushed with gas containing 1% O_2, 5% CO_2, and 94% N_2 for 5 minutes, and incubated in this environment at 37˚C for indicated times.

HIF Peptide Prolyl Hydroxylation Assay

Extract was prepared by harvesting isolated tissue in assay buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol) at 4°C using a Dounce homogenizer. The lysate was centrifuged at 20,000x***g*** for 15 min. The supernatant (75 μg) was used as the source of enzyme in the ^{35}S-labelled pVHL pulldown assay, as described previously by Bruick et al. Analysis of activity was measured by scintillation counting or autoradiography after gel electrophoresis on a 10-20% Novex Tris Glycine gradient gel (Invitrogen).

qPCR mRNA analysis

Isolated tissue was treated with RNAlater (Qiagen) to stabilize total RNA. Total RNA was then isolated using the RNeasy Mini Kit (Qiagen). Total RNA (5 μg) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). Rat specific primers which spanned an exon-exon boundary were used. For quantitative PCR, the iQ SYBR Green Supermix (Bio-Rad) and MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) was used. The following primers were used: for rat HPH-1 (GenBank accession no. NM_019371), forward = 5'- GGCCGCTGTATCACCTGTAT - 3', reverse = 5'- TTCTGCCCTTTCTTCAGCAT -3'; for rat HPH-2 (GenBank accession no. NM_178334), forward = 5'- TACGTCCGTCACGTCGATTA -3', reverse = 5'- TTGGGTTCAATGTCAGCAAA - 3'; for rat HPH-3 (GenBank accession no. NM_001004083), forward = 5'- CTGGCAACTACGTCATCAA -3', reverse = 5'- GATTGTCAACATGCCTCACG -3'; for rat CA9 (GenBank accession no. XM233380), forward = 5' - CCTGCTGAGATCCATGTGGT - 3', reverse = 5' - TGGGACAGCAACTGTTCGTA - 3'; for rat HO-1 (GenBank accession no. NM_012580), forward = 5' - AAGAGGCTAAGACCGCCTTC - 3', reverse = 5' - CCTCTGGCGAAAGAAACTCTG - 3'. Primer temperature, concentration, and cDNA dilution optimizations were conducted before analysis and specific single-band amplification was verified through multicomponent analysis and sequencing of amplified products.

In Situ Hybridization

Cryosections of 16 μM thickness were cut on a cryostat and collected on Fisherbrand Superfrost Plus Slides (Fisher Scientific). Sections were stored at -70 degrees Celsius

until day of hybridization. DNA templates for the generation of RNA in situ hybridization probes were generated by PCR using the HotStarTaq PCR Kit (Qiagen). Antisense probe templates were generated by incorporating a T7 sequence to the 3' primer. Sense probe templates were generated by incorporating a SP6 sequence to the 5' primer. The following primer sequences for DNA template construction were used: for rat HPH-1 (GenBank accession no. NM_019371), forward = 5'-TGCCCTCCAGACACTCTCTT -3', reverse = 5'- AACACTCTCGGGTCTGTGCT -3'; for rat HPH-2 (GenBank accession no. NM_178334), forward = 5'-TGGTCAGCCAGAAGAGTGAC -3', reverse = 5'- GGCTTGAGTTCAACCCTCAC -3'; for rat HPH-3 (GenBank accession no. NM_001004083), forward = 5' -AGGCCCTGAATCAAGCTCTC -3', reverse = 5'- GGATTGTCAACATGCCTCACG -3. DNA probe template sequences were confirmed by DNA sequencing using BigDye Terminator Kit (Applied Biosystems). RNA probes were then synthesized using the MAXIscript In vitro Transcription Kit (Ambion) with the appropriate T7 or SP6 enzyme and ^{35}S-UTP nucleotides (Amerisham Biosciences). Unincorporated nucleotides were removed with NucAway Spin Columns (Ambion). HPH-1, HPH-2, and HPH-3 in situ probe lengths were 1081, 529, and 903, nucleotides respectively. RNA probe size was confirmed by gel electrophoresis and visualization. In situ hybridization was performed as previously described (Xing et al., 1997). Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, washed in PBS, then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min to minimize nonspecific hybridization of the probes. After dehydration with increasing concentrations of ethanol, the sections were delipidated in chloroform for 5 min, rinsed in

ethanol and air dried. Brain sections were hybridized overnight at 56 degrees Celsius with 1 x 10^6 cpm of ^{35}S-UTP labeled riboprobe in 100 ml of hybridization buffer containing 20 mM Tris-HCI (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM EDTA (pH 8.0), 1 X Denhardt's solution, 250 mg/ml yeast tRNA, 250 mg/ml total RNA, 100 mg/ml salmon sperm DNA and 10% dextran sulfate, 100 mM dithiothreitol, 0.1% SDS, and 0.1% sodium thiosulfate. Sections then were rinsed four times for 5 min each in 4 X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) and treated with 20 mg RNase A (Boehringer Mannheim) per ml of 0.5 M NaCl/1 mM EDTA/10 mM tris, pH 8, for 30 min at 24 degrees Celsius. After rinsing for 5 min each in 1X, 0.5X and 0.1X SSC at 24 degrees Celsius, slides were washed in 0.1 X SSC twice for 30 min each at 65 degrees Celsius. The final rinsing was performed with increasing concentrations of ethanol (70%, 90% and 95%) containing 300 mM ammonium acetate. Slides were air-dried and exposed to Kodak Min-R film for 4 days at room temperature.

Western Blot Analysis

Whole cell extracts were prepared by lysing pelleted cells in RIPA buffer (0.1% SDS, 1% Nonidet P40, 5 mM EDTA, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris/HCl, freshly supplemented with 2 mM dithiothreitol and protease inhibitors) for 30–60 min on ice. Lysates were centrifuged at 16,000x***g*** for 10 min (4°C), the supernatant was subjected to electrophoresis in Novex 4-12% or 10–20% Tris/glycine precast gels (Invitrogen), transferred on to nitrocellulose, and blotted using the polyclonal antibodies as indicated below.

Antibodies and Chemical Reagents

Rabbit polyclonal anti HPH-1 and HPH-3 antibodies were NB 100-139 (Novus Biologicals), and NB100-310 **(**Novus Biologicals), respectively. Mouse monoclonal anti FIH-1 antibody was NB100-428A (Novus Biologicals). Mouse monoclonal anti β-actin antibody was ab6276-100 (Abcam). N-oxalylglycine was O1141 (Frontier Scientific).

RESULTS

Rat Brain Regions Display Differential Tissue HIF Hydroxylation Activity

Our study was initiated by a report demonstrating the presence of HPH expression in the rat brain (Freeman, Hasbani et al. 2003). We confirmed the presence of mRNA transcripts of all HPH homologues in the rat brain by RT-PCR (data not shown). To dettermine HIF prolyl hydroxylation activity level in various rat brain regions, we utilized an *in vitro* enzymatic assay coupled to the pulldown of radioactively-labelled pVHL as previously described (Dalgard, Lu et al. 2004). Cellular extracts from whole rat brain were prepared and tested to assess proper responsiveness of enzymatic activity to known activators and inactivators of prolyl hydroxylation (Figure 2a). Minimal enzymatic activity was observed in reactions without cellular extracts. In reactions with extracts as the enzyme source, minimal enzymatic activity was also observed in the absence the reaction substrate 2-oxoglurate and the cofactor ferrous iron. Enzymatic activity was significantly increased in reactions with 2-oxoglutarate and ferrous iron. This activity was further increased with the addition of the cofactor ascorbate. Reassuringly, the known competitive inhibitor, N-oxalylglycine, reduced enzymatic activity to minimal levels. These appropriate pharmacological responses found in the hydroxylation assay help validate the following studies of enzymatic activity in brain tissue. We used cellular extracts prepared from isolated regions of young rat brains to compare the prolyl hydroxylase capacity of the brain stem, cerebellum, cerebral cortex, and hippocampus. Differences in HIF prolyl hydroxylase activity determined by the binding of [^{35}S]-labelled pVHL was visible on an autoradiograph when using enzymatic source from various brain regions (Figure 2b). These experiments reflect a total

maximum capacity of HIF prolyl hydroxylation and not *in vivo* hydroxylation activities due to the use of excess substrates (HIF peptide and 2-oxoglutarate) and cofactors (ferrous iron and ascorbate). To optimize the ability to distinguish differences between various rat brain regions in their HIF hydroxylation capacity, we performed the hydroxylation assay at various time points using extracts from hippocampus and cerebellum as enzymatic source (Figure 2c). In all time points performed, statistically significant differences ($p<0.05$, student's t-test) were found between hippocampus and cerebellum enzymatic capacity. A reaction time of 20 minutes was chosen for the remainder of the study based on the previous results. Figure 3 shows the HIF hydroxylation capacity of various rat brain regions and capacity changes in response to hypoxia. Cellular extracts were obtained from the brain regions of several animals untreated (n=6) and treated (n=5) with hypoxia (8% O_2) for 8 hours. Two ages of animals were studied, 4 week old rat pups and 8 week old rat young adults. In the 4 week old rat pups, cerebellum displayed the highest total hydroxylation capacity, followed by hippocampus, brain stem, and cortex. These differences were signficiant (ANOVA, $p < 0.05$). Additionally, exposure to hypoxia increased potential HIF hydroxylation activity in the brain stem, cortex, and hippocampus ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). Surprisingly, hydroxylation activity did not increase in extracts obtained from cerebellum. In the 8 week old rat pups, cerebellum again displayed the highest total hydroxylation capacity. However, in contrast to the results found in 4 week old rats, brain stem, cortex, and hippocampus all possess similar levels of hydroxylation activity. Additionally, hypoxia treatment did not increase activity in any of the studied brain regions. These results suggests a difference in the hypoxic responsiveness of HPH-

mediated HIF hydroxylation capacity in 4 week old versus 8 week old rats, as well as the presence of differential hydroxylation capacity in the these rat brain regions in younger rat pups.

Rat Brain Displays Differential HPH Gene Expression Patterns and Regulation

Our findings of differential HIF hydroxylation capacity and hypoxic induction in various rat brain regions led us to investigate the expression patterns of the HPH homologues in the rat brain. We studied whether the mRNA expression patterns of the HPH homologues could explain the difference in total potential hydroxylation activity found in rat brain regions. We determined HPH homologue mRNA expression level in rat brain stem, cerebellum, and cerebral cortex by quantitative PCR in 4 week old rat pups. Total RNA from these brain regions were isolated from the same animal tissue analyzed for HIF prolyl hydroxylation activity in the previous section. In untreated animals, we found that HPH-1 mRNA levels were highest in the brain stem, followed by cerebellum. In agreement with the hydroxylation data, HPH-2 and HPH-3 mRNA levels were highest in the cerebellum, followed by the brain stem. Lowest mRNA levels of all 3 of the HPH homologues were found in the cerebral cortex, which correlates with the lowest level of enzymatic activity found among the 4 brain regions investigated. In cell culture studies, HPH-1 and HPH-2 mRNA induction by hypoxia is well established. We witnessed an increase of brain stem HPH-1 mRNA expression of HPH-1 in hypoxia treated animals as compared to untreated controls ($p < 0.01$, student's t-test). However, significant increases were not observed in cerebellum or cortex. Induction of HPH-2 mRNA levels were also found in the brain stem and cerebellum ($p < 0.01$ and $p < 0.05$, respectively), but not in the cortex. Not surprisingly, HPH-3 mRNA levels did not change significantly

in hypoxia treated animals as compared to untreated controls. A control HIF-1 target gene, carbonic anhydrase IX, was found to be induced by hypoxia in the brain stem and cerebellum, but not the cortex, in lines with previously reported expression data of other HIF-1 target genes (Colombrita, Calabrese et al. 2003). We also investigated HPH-1 protein expression from whole cell extracts of untreated and hypoxia (0.1% CO for 8 hours) treated tissue. HPH-1 is known to exist as two forms in rat cells, a full length ~40 kDa transcript and a smaller truncated ~30 kDA transcript which is localized to the mitochondria (Freeman, Hasbani et al. 2003). Utilizing western blot analysis, we observed both of these protein forms in extracts prepared from rat brain tissues (Figure 7). In agreement with HPH-1 mRNA expression data, protein levels of the full level HPH-1 transcript were highest in the brain stem, while lower levels were present in the cerebellum and cortex. Surprisingly, full length HPH-1 protein levels did not visibly increase under hypoxia in brain stem extracts, but were seen in extracts from the cerebellum. However, mitochondrial 30 kDa HPH-1 protein induction by hypoxia was observed in extracts prepared from all tissues investigated.

Level of HPH mRNA Expression Varies Between Cell Type in Rat Embryonic Primary Cultures

HPH mRNA expression has been studied in human (Epstein, Gleadle et al. 2001; Berra, Benizri et al. 2003; Metzen, Berchner-Pfannschmidt et al. 2003) and rat permanent cell lines (D'Angelo, Duplan et al. 2003) as well as primary human cells (Marxsen, Stengel et al. 2004), but not primary rat brain cells. We cultured primary cortical astrocytes and neurons from E17 embryonic rats to establish that expression and regulation of the HPH homologues resembles that found in previous work. Rat primary cell cultures were

grown in normoxic conditions or subjected to hypoxia and HPH expression was analyzed by qPCR. All results were normalized to HPRT mRNA levels, which were found to be equal in both astrocytes and neurons. We found that HPH-1 and HPH-2 mRNA regulation by hypoxia was present in both rat primary cortical astrocytes and neurons (Figure 8). In agreement with studies in other cell lines, HPH-3 mRNA expression demonstrated no regulation by hypoxia. However, it was interesting to find that HPH-3 mRNA expression was significantly higher in primary cortical astrocytes compared to cortical neurons ($p < 0.01$). The expression of a known HIF-1 target gene, heme oxygenase (HO-1), demonstrated an appropriate induction in response to hypoxia in the same samples.

Level of HPH-1 mRNA Expression is Altered in Rat Gliomas

We also conducted comparative analysis of HPH expression between rat embryonic primary cortical astrocytes and C6 rat glioma cells. Similar to findings from comparative analysis of cortical astrocytes and neurons, as well as previous studies, we observed HPH-1 and HPH-2 mRNA induction by hypoxia in C6 rat glioma cells (Figure 9). Appropriately, HPH-3 mRNA expression did not change in response to hypoxia in C6 rat glioma cells. However, we did find that HPH-1 mRNA levels were significantly higher in primary cortical astrocytes as compared to C6 rat glioma cells in both normoxic and hypoxia treated conditions ($p < 0.01$ each). We investigated this difference in HPH-1 expression by western blot analysis. HPH-1 protein levels were detectable and demonstrated hypoxia-mediated induction in whole cell extracts from primary cortical astrocytes. In contrast, C6 rat glioma cells did not display significant increase in HPH-1 protein levels when treated with hypoxia for 16 hours.

DISCUSSION

The HIF prolyl hydroxylases (HPH-1, HPH-2, and HPH-3) are the main regulatory enzymes for controlling HIF-1α protein levels and HIF-1 activity. Since these enzymes require molecular oxygen to catalyze the hydroxylation of critical HIF-1α proline residues, they have become the "elusive" oxygen sensors in the HIF signaling pathway. We set out to investigate the expression properties of the HPH homologues in the rat brain to better understand oxygen sensing and hypoxia adaptation in central nervous tissue. We demonstrate by qPCR and in situ hybridization analysis that rat brain regions differ in HPH mRNA expression for each of the three homologues. Especially notable is the high HPH-2 and HPH-3 and moderate HPH-1 expression levels found in cerebellum compared to brain stem and cortex. These expression differences could directly contribute to the high HIF hydroxylation capacity also found in cerebellum compared to other brain regions studied. We also found that HPH-2 and CA9, both HIF-1 target genes, displayed expression levels that were highest in the cerebellum, followed by brain stem, with lowest levels in the cortex. Interestingly, another study of mRNA expression of a HIF-1 target gene, heme oxygenase-1, led to the identical relative expression patterns in the rat brain (Colombrita, Calabrese et al. 2003).

A significant decrease in HPH-1 expression was discovered during comparative analysis of C6 glioma cells to rat primary cortical astrocytes. It has been appreciated that increased HIF-1α protein levels are commonly found in several cancer types (Zhong, De Marzo et al. 1999; Talks, Turley et al. 2000) and is associated with increased patient mortality (Beasley, Leek et al. 2002). Intratumoral hypoxia (Harris 2002), oncogenic alterations in signalling (Maxwell, Wiesener et al. 1999), and changes in glycolytic

metabolism (Lu, Forbes et al. 2002) have all been demonstrated to cause HIF-1α overexpression in cancer cells. A change in HPH expression or activity as a cause of HIF-1α overexpression has not been explored in cancer cells. The finding that C6 gliomas have significantly decreased levels of HPH-1 in both normoxic and hypoxic conditions may explain a higher level of HIF-1 signaling in these cells as compared to normal cortical astrocytes.

In all experiments of this study, HPH-3 was found not to be hypoxia induced, which is in agreement with all current literature. HPH-3 did demonstrate differential expression levels in the rat brain and has been shown to have tissue-specific expression patterns in the mouse (Lieb, Menzies et al. 2002) and human (Hirsila, Koivunen et al. 2003). Additionally, we did witness a significant level of mRNA expression different between rat primary astrocytes and cortical neurons. The underlying reason for this difference remains unknown, but since other studies have found HPH-3 expression difference in normal and cancerous renal epithelium (Jiang, Zhang et al. 2003), as well as evidence for HPH-3 regulation by estrogen (Seth, Krop et al. 2002; Appelhoff, Tian et al. 2004), it is clear that a non-hypoxia regulatory mechanism exists and awaits more detailed investigation.

In conclusion, we have provided the first detailed description of HPH expression in the mammalian brain. These findings provide an understanding to the potential signaling capacity of the HPH enzymes in brain cells. However, an analysis of the regulatory transcription factors and microenviromental conditions which drive HPH gene transcription in brain tissue and cells is clearly necessary to comprehend why these differences exist. Additionally, it is obvious to these authors that further work in

identifying additional protein targets of the HPH homologues is paramount to the understanding the functional relevance of distinct, yet overlapping expression in mammalian brain tissue.

REFERENCES

Appelhoff, R. J., Y. M. Tian, et al. (2004). "Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor." J Biol Chem 279(37): 38458-65.

Beasley, N. J., R. Leek, et al. (2002). "Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients." Cancer Res 62(9): 2493-7.

Berra, E., E. Benizri, et al. (2003). "HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia." Embo J 22(16): 4082-90.

Bruick, R. K. and S. L. McKnight (2001). "A conserved family of prolyl-4-hydroxylases that modify HIF." Science 294(5545): 1337-40.

Colombrita, C., V. Calabrese, et al. (2003). "Regional rat brain distribution of heme oxygenase-1 and manganese superoxide dismutase mRNA: relevance of redox homeostasis in the aging processes." Exp Biol Med (Maywood) 228(5): 517-24.

D'Angelo, G., E. Duplan, et al. (2003). "Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation." J Biol Chem 278(40): 38183-7.

Dalgard, C. L., H. Lu, et al. (2004). "Endogenous 2-oxoacids differentially regulate expression of oxygen sensors." Biochem J 380(Pt 2): 419-24.

Dijkhuizen, R. M., S. Knollema, et al. (1998). "Dynamics of cerebral tissue injury and perfusion after temporary hypoxia-ischemia in the rat: evidence for region-specific sensitivity and delayed damage." Stroke 29(3): 695-704.

Epstein, A. C., J. M. Gleadle, et al. (2001). "C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation." Cell 107(1): 43-54.

Freeman, R. S., D. M. Hasbani, et al. (2003). "SM-20, EGL-9, and the EGLN family of hypoxia-inducible factor prolyl hydroxylases." Mol Cells 16(1): 1-12.

Harris, A. L. (2002). "Hypoxia--a key regulatory factor in tumour growth." Nat Rev Cancer 2(1): 38-47.

Hirsila, M., P. Koivunen, et al. (2003). "Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor." J Biol Chem 278(33): 30772-80.

Ivan, M., K. Kondo, et al. (2001). "HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing." Science 292(5516): 464-8.

Jaakkola, P., D. R. Mole, et al. (2001). "Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation." Science 292(5516): 468-72.

Jiang, Y., W. Zhang, et al. (2003). "Gene expression profiling in a renal cell carcinoma cell line: dissecting VHL and hypoxia-dependent pathways." Mol Cancer Res 1(6): 453-62.

Koong, A. C., N. C. Denko, et al. (2000). "Candidate genes for the hypoxic tumor phenotype." Cancer Res 60(4): 883-7.

Lieb, M. E., K. Menzies, et al. (2002). "Mammalian EGLN genes have distinct patterns of mRNA expression and regulation." Biochem Cell Biol 80(4): 421-6.

Lu, H., R. A. Forbes, et al. (2002). "Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis." J Biol Chem 277(26): 23111-5.

Marxsen, J. H., P. Stengel, et al. (2004). "Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases." Biochem J 381(Pt 3): 761-7.

Maxwell, P. H., M. S. Wiesener, et al. (1999). "The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis." Nature 399(6733): 271-5.

Metzen, E., U. Berchner-Pfannschmidt, et al. (2003). "Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing." J Cell Sci 116(Pt 7): 1319-26.

Metzen, E. and P. J. Ratcliffe (2004). "HIF hydroxylation and cellular oxygen sensing." Biol Chem 385(3-4): 223-30.

Schofield, C. J. and P. J. Ratcliffe (2004). "Oxygen sensing by HIF hydroxylases." Nat Rev Mol Cell Biol 5(5): 343-54.

Seth, P., I. Krop, et al. (2002). "Novel estrogen and tamoxifen induced genes identified by SAGE (Serial Analysis of Gene Expression)." Oncogene 21(5): 836-43.

Talks, K. L., H. Turley, et al. (2000). "The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages." Am J Pathol 157(2): 411-21.

Wang, G. L., B. H. Jiang, et al. (1995). "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension." Proc Natl Acad Sci U S A 92(12): 5510-4.

Wykoff, C. C., C. W. Pugh, et al. (2000). "Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling." Oncogene 19(54): 6297-305.

Yu, F., S. B. White, et al. (2001). "HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation." Proc Natl Acad Sci U S A 98(17): 9630-5.

Zhong, H., A. M. De Marzo, et al. (1999). "Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases." Cancer Res 59(22): 5830-5.

FIGURE LEGENDS

Figure 1. Nomenclature and Physical Characteristics of the rat HIF Prolyl Hydroxylase Homologues. HPH nomenclature and its association with other nomenclatures for these homologues are shown. The length of the HPH homologues in amino acids and mass in kilo Daltons is also shown. Location of the prolyl hydroxylation catalytic domain in a linear structure of the homologues is depicted.

Figure 2. HIF-1α prolyl hydroxylase activity from young adult rat brain extracts. Eight week old rat brain regions were isolated and protein extracts were prepared. 75 μg of extract was used to hydroxylate a HIF-1α ODD peptide. HPH enzymatic activity was monitored by the ability to pulldown *in vitro* translated ^{35}S-pVHL. A) Activity from rat whole brain extract displays appropriate responses to the known substrate 2-oxoglutarate and the known cofactor ascorbate as well as the 2-oxoglutarate competitive inhibitor, N-oxalylglycine. Assay time was 25 minutes. B) Several isolated rat brain regions were examined for activity. Captured ^{35}S-pVHL was monitored by autoradiography following gel electrophoresis. Assay time was 30 minutes. C) Enzymatic activity from cerebellum and hippocampus was monitored after various reaction times. Mean and standard deviation is reported from multiple samples (n=4).

Figure 3. HIF-1α prolyl hydroxylase activity in various brain region extracts. Four and eight week old rats were untreated or treated with hypoxia (8% O_2) for 8 hours. Brain stem, cerebellum, cortex, and hippocampus protein extracts were isolated immediately after treatment. HPH enzymatic activity was monitored by the ability to pulldown *in vitro* translated ^{35}S-pVHL. The assay time was 20 minutes using 70 μg of

extract. Mean and standard deviation is reported from multiple samples (n=6 for normoxia, n=5 for hypoxia).

Figure 4. qPCR analysis of HPH mRNA expression in young adult rat brain regions. Four week old male Wistar Hanover rats were untreated or treated with hypoxia (8% O_2) for 8 hours. Rat brain regions were isolated and placed into RNAlater (Qiagen) overnight at 4°C. Total RNA from tissues was isolated using the RNeasy Mini Kit (Qiagen), cDNA was generated, and qPCR analysis was performed. Raw expression levels from triplicate readings were first normalized to HPRT. Results are reported as fold change normalized by expression level of the gene found in normoxic cortex. Mean and standard error are reported from multiple samples (n=5).

Figure 5. In situ hybridization analysis of HPH mRNA expression in young rat tissues. A) In situ hybridization autoradiographs of HPH-1, HPH-2, and HPH-3 target genes from whole rat embryos. HPH homologues are expressed in various tissues with some tissue specificity. B) In situ hybridization autoradiographs of HPH-1, HPH-2, and HPH-3 genes from young rat heart (left) and testis (right) tissues. Four week old male Wistar Hanover rats were untreated or treated with hypoxia (8% O_2) for 8 hours. HPH homologue probes showed appropriate tissue specificity. HPH-1 displayed greater expression in heart tissue, HPH-2 displayed expression in both heart and testes, while HPH-3 displayed highest expression in the testes.

Figure 6. In situ hybridization analysis of HPH mRNA expression in young rat brain. Four week old male Wistar Hanover rats were untreated or treated with hypoxia (8% O_2) for 8 hours. A) In situ hybridization autoradiographs of HPH-1, HPH-2, and HPH-3 target genes from sagittal sections of young rat brain. Untreated tissue (top) and

treated tissue (bottom) were hybridized simultaneously. Note the increase in HPH-1 expression in treated brain stem compared to controls. An increase of HPH-2 mRNA expression was observed in several regions of treated brain tissue compared to controls. HPH-3 did not display significant changes in mRNA expression between treated brain tissue and untreated controls. B) In situ hybridization autoradiographs of HPH-1, HPH-2, and HPH-3 target genes from coronal sections of young rat cerebellum and brain stem tissue. Note the increase in HPH-1 expression in treated brain stem compared to controls. An increase of HPH-2 mRNA expression was observed in both the cerebellum and brain stem of treated brain tissue compared to controls. HPH-3 did not display significant changes in mRNA expression between treated brain tissue and untreated controls.

Figure 7. Western blot analysis of HPH protein expression in young adult rat brain. Eight week old rats were treated with either room air or 0.1% CO for 8 hours. Rat brain regions were isolated and whole cell extracts were prepared using T-PER Tissue Protein Extraction Reagent (Pierce). Gel electrophoresis in a 10-20% gradient gel was performed on extracts before transfer to nitrocellulose. Western blot analysis was performed using HPH-1 and HPH-3 polyclonal antibodies from Novus Biologicals.

Figure 8. qPCR analysis of HPH mRNA expression in rat primary cortical astrocytes and neurons. Astrocytes and neurons were isolated from E17 rat cortex and cultured for 7 days. Cells were then cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 16 hours before total RNA was isolated. After generation of cDNA, qPCR analysis was conducted using rat specific primers spanning an exon-exon boundary. Raw expression levels from triplicate readings were first normalized to the housekeeping gene HPRT. Results are reported as fold change normalized by expression level of the gene

found in normoxic cortical neurons. Mean and standard error are reported from multiple samples (n=3).

Figure 9. qPCR analysis of HPH mRNA expression in rat primary cortical astrocytes and C6 gliomas. Astrocytes were isolated from E17 rat cortex. Astrocytes and C6 glioma cells were then cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 16 hours before total RNA was isolated. After generation of cDNA, qPCR analysis was conducted using rat specific primers spanning an exon-exon boundary. Raw expression levels from triplicate readings were normalized to the housekeeping gene HPRT. Results are reported as fold change normalized by expression level of the gene found in normoxic C6 gliomas. Mean and standard error are reported from multiple samples (n=4).

Figure 10. Western blot analysis of HPH-1 protein expression in rat primary cortical astrocytes and C6 gliomas. Astrocytes were isolated from E17 rat cortex. Astrocytes and C6 glioma cells were then cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 16 hours before whole cell extracts were isolated. Gel electrophoresis in a 4-12% gradient gel was performed on extracts before transfer to nitrocellulose. Western blot analysis was performed using a HPH-1 polyclonal antibody from Novus Biologicals.

Figure 1.

HPH	EGLN	PHD	Length	MW	Schematic
1	3	3	355	39.8	
2	1	2	222	25.0	
3	2	1	415	44.7	

Figure 2.

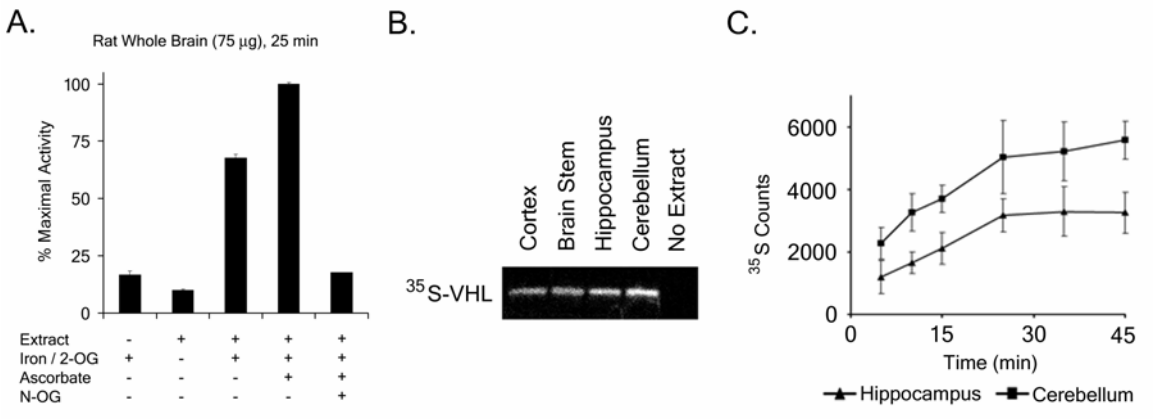

A.
Rat Whole Brain (75 µg), 25 min
% Maximal Activity
100
75
50
25
0
Extract - + + + +
Iron / 2-OG + - + + +
Ascorbate - - - + +
N-OG - - - - +
B.
Cortex
Brain Stem
Hippocampus
Cerebellum
No Extract
35S-VHL
C.
35S Counts
6000
4000
2000
0
0
15
30
45
Time (min)
Hippocampus
Cerebellum

Figure 3.

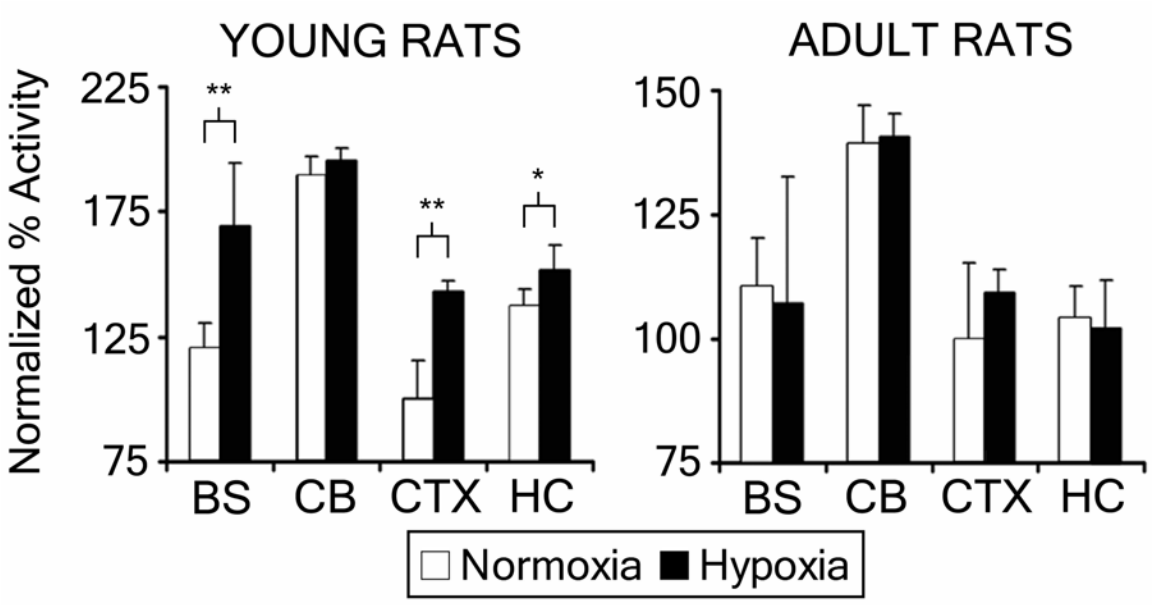

YOUNG RATS
ADULT RATS
Normalized % Activity
225
175
125
75
150
125
100
75
**
**
*
BS
CB
CTX
HC
Normoxia
Hypoxia

Figure 4.

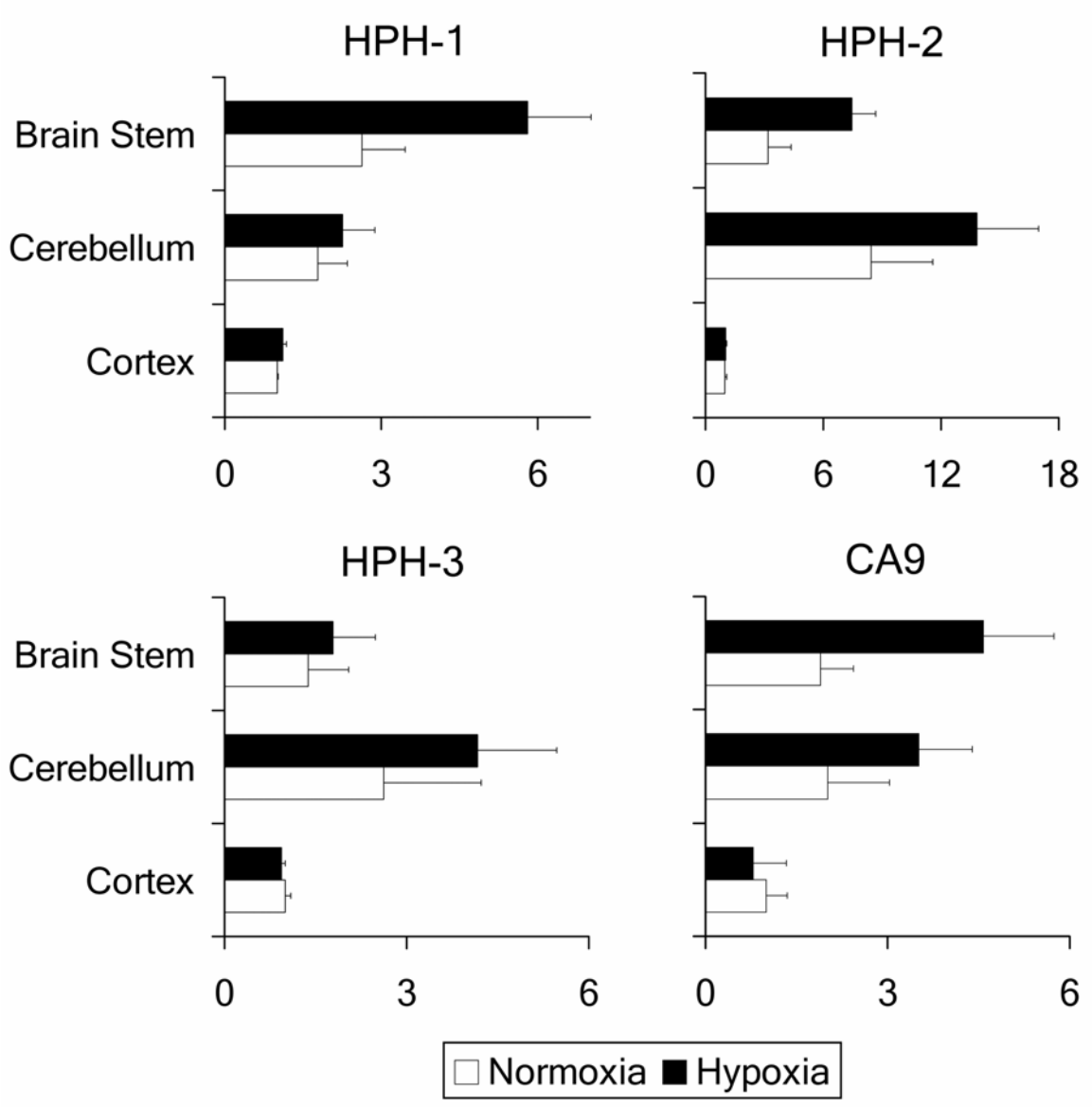

HPH-1
HPH-2
HPH-3
CA9
Brain Stem
Cerebellum
Cortex
0
3
6
12
18
Normoxia
Hypoxia

Figure 5.

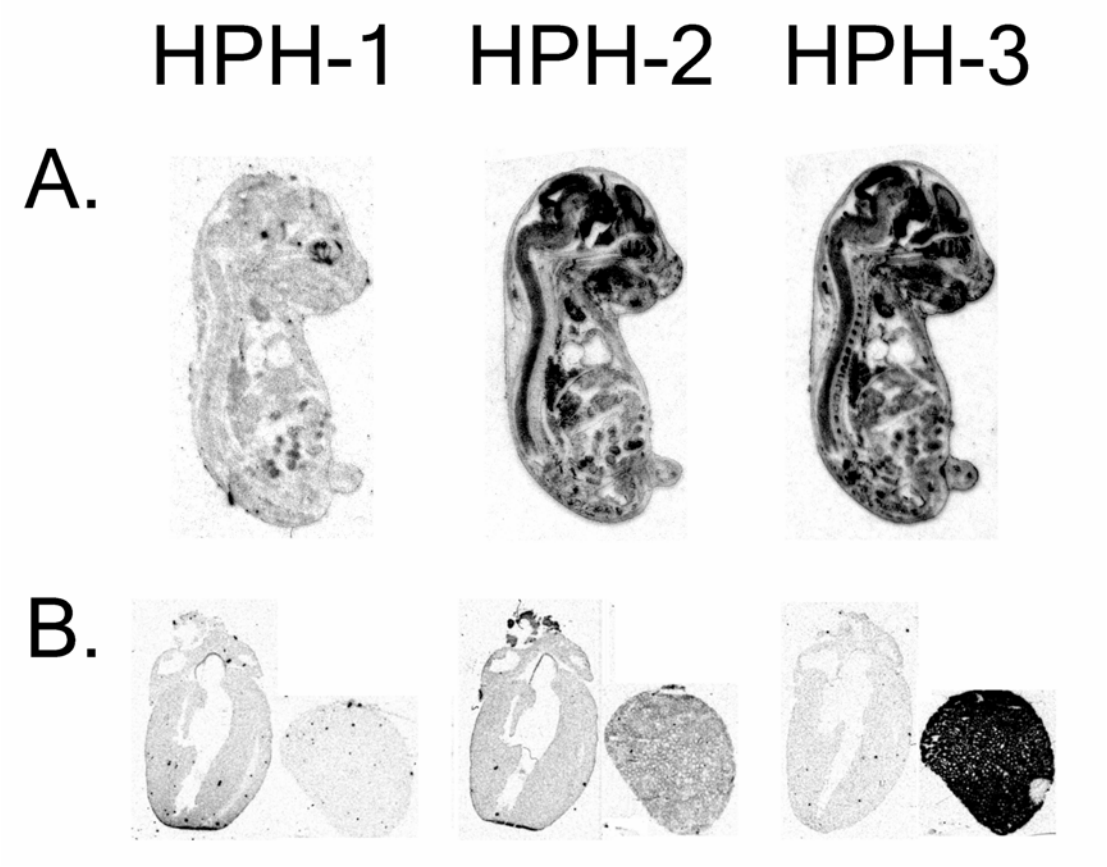


Figure 6.

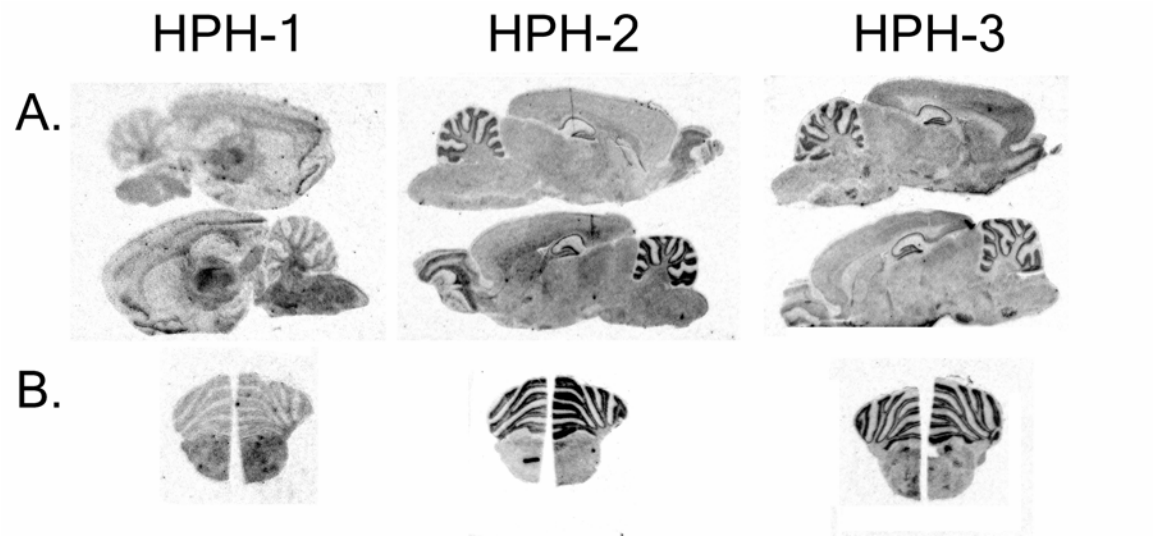


Figure 7.

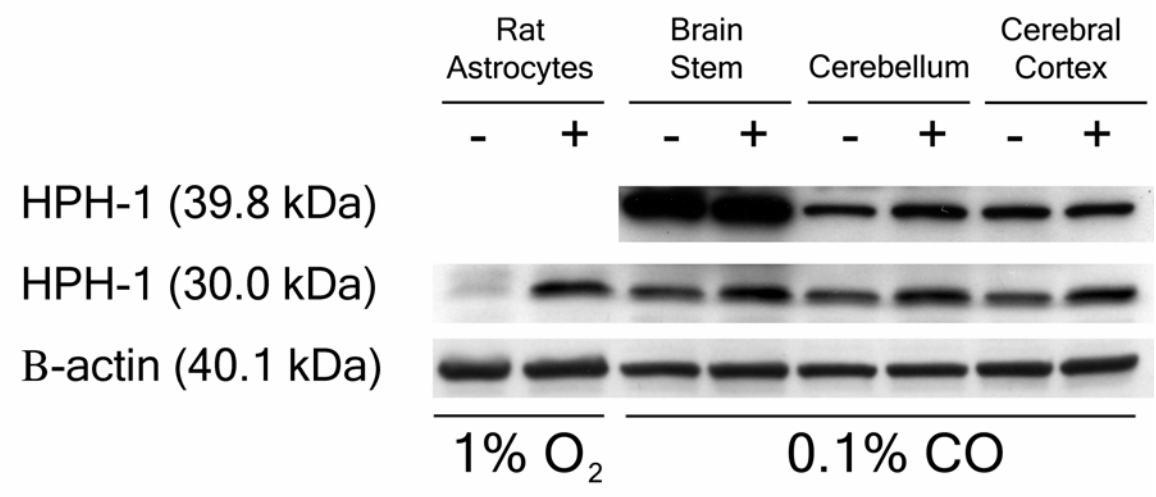

Rat Astrocytes
Brain Stem
Cerebellum
Cerebral Cortex
- + - + - + - +
HPH-1 (39.8 kDa)
HPH-1 (30.0 kDa)
B-actin (40.1 kDa)
1% O2
0.1% CO

Figure 8.

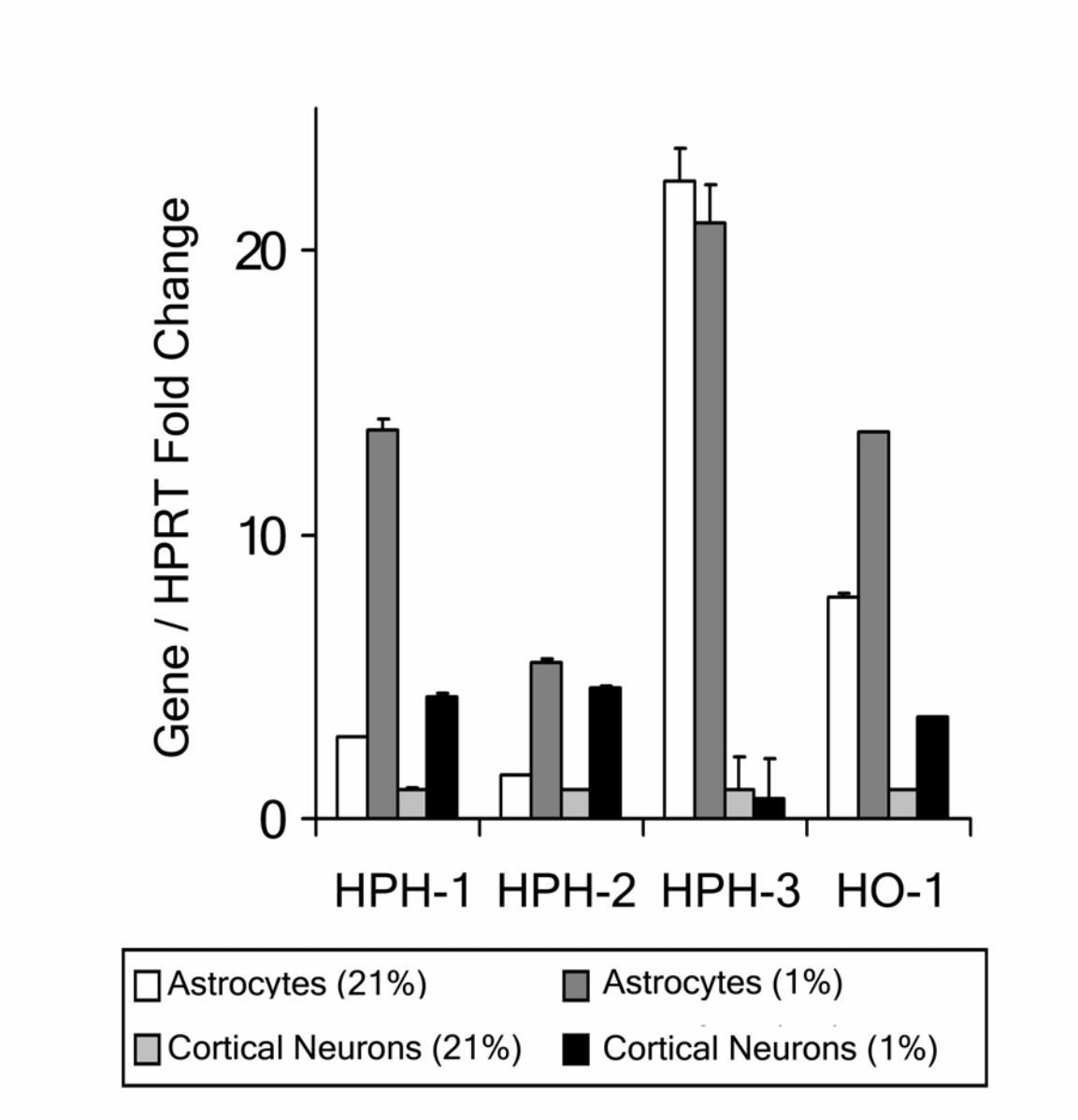

Gene / HPRT Fold Change
20
10
0
HPH-1
HPH-2
HPH-3
HO-1
Astrocytes (21%)
Astrocytes (1%)
Cortical Neurons (21%)
Cortical Neurons (1%)

Figure 9.

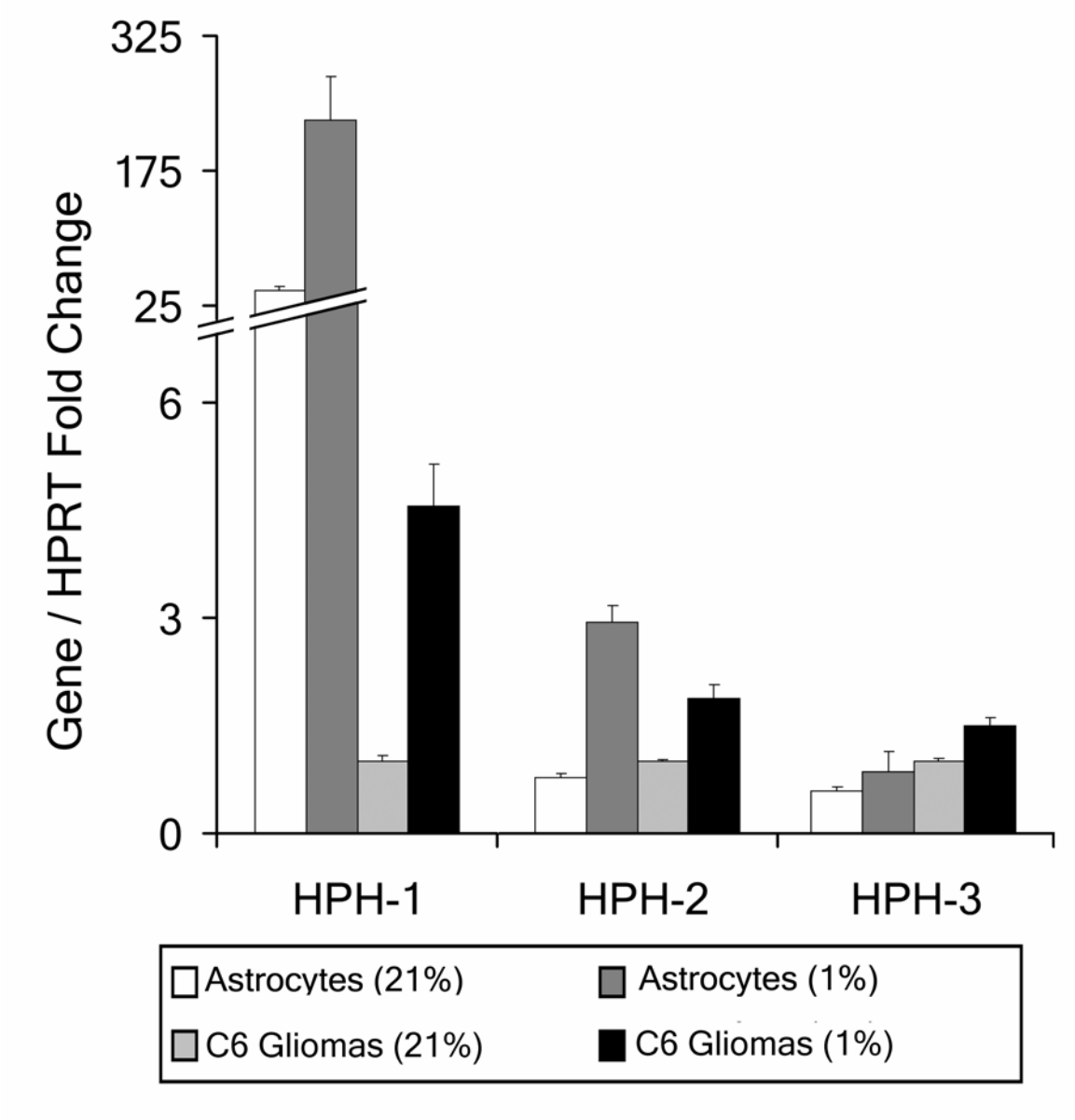
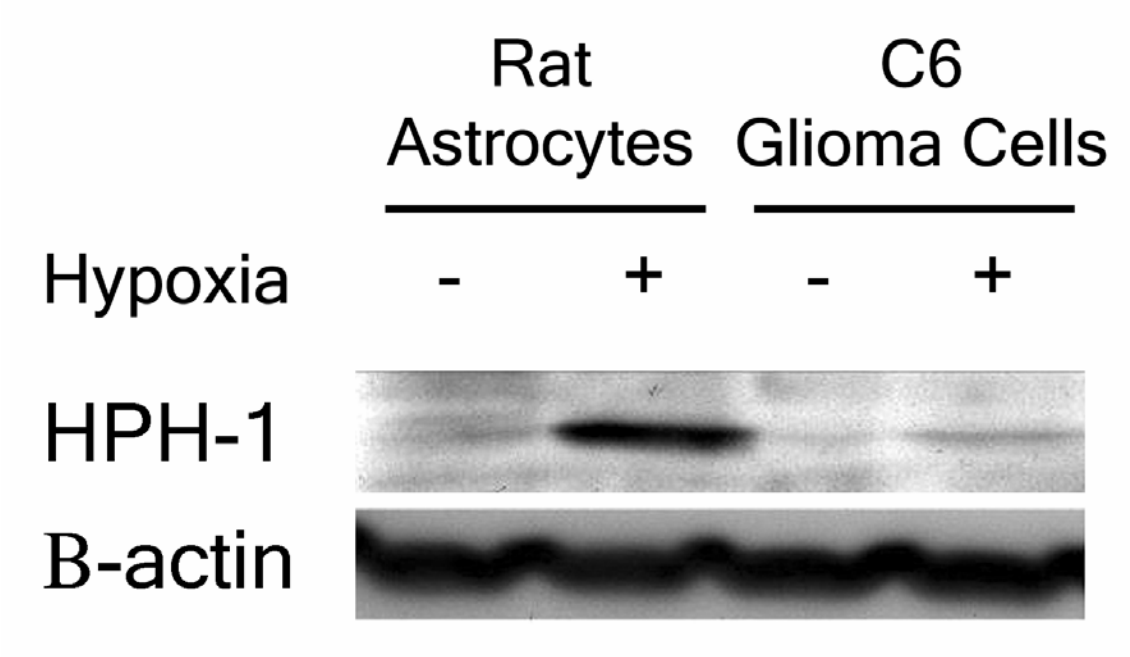

325
175
25
6
3
0
Gene / HPRT Fold Change
HPH-1
HPH-2
HPH-3
Astrocytes (21%)
Astrocytes (1%)
C6 Gliomas (21%)
C6 Gliomas (1%)

Figure 10.



DISCUSSION

The objective of this thesis work was to investigate the central nervous system biology of the HIF prolyl hydroxylases (HPH-1, HPH-2, HPH-3). We determined that HIF-1 mediated expression of HPH-1 and HPH-2 oxygen sensors constitute a feedback loop for modulating hypoxic gene expression in human gliomas cells. We also determined that the HPH oxygen sensors are inhibited by glycolytic metabolites and in turn, regulate genetic adaptations in response to changes in energy metabolism. Finally, we describe the HPH oxygen sensors anatomical and/or cellular expression patterns in the rat central nervous system.

HPH-1 and HPH-2 Upregulation by HIF-1 Activation in Human Glioma Cells Constitutes a Negative Feedback Loop

At this initiation of these studies, it was appreciated that HPH-1 and HPH-2 mRNA expression is inducible by hypoxia in human permanent cell lines (Epstein, Gleadle et al. 2001; Metzen, Berchner-Pfannschmidt et al. 2003). Additionally, HIF hydroxylation activity was known to increase after exposure to hypoxia. We proved that these HPH-related properties were also found in human gliomas, rat primary brain cells, and rat brain tissue. In human gliomas, HPH-1 and HPH-2 mRNA, protein level and HPH enzymatic activity increase significantly with increasing duration of hypoxia exposure (Paper 1, Figure 1). These increases are associated with an increase in the rate of HIF-1α decay following reoxygenation after exposure to hypoxia (Paper 1, Figure 1e). The induction of HPH-1 and HPH-2 by hypoxia is not unique in human gliomas. We

found that several hypoxia "mimetics" were able to induce HPH-1 and HPH-2 to similar levels found in hypoxia-treated cells (Paper 1, Figure 3a). In studies utilizing rat brain tissue, we found HPH-1 and HPH-2 to be hypoxia-inducible in several brain regions. This finding is the first evidence that HPH expression is regulated *in vivo* by hypoxia. qPCR (Paper 3, Figure 4) and *in situ* hybridization analysis (Paper 3, Figure 6) demonstrated increased mRNA expression in the brain stem for HPH-1 and in all brain regions for HPH-2. Protein expression of the mitochondrial isoform of HPH-1 is induced by hypoxia in rat brain stem, cerebellum, and cerebral cortex (Paper 3 Figures 7). HPH-1 and HPH-2 mRNA expression is also hypoxia-inducible in rat primary cortical astrocytes, rat primary cortical neurons, and C6 gliomas (Paper 3 Figures 8-10). Two possible functions for hypoxia induced HPH expression exist. First, increased HPH activity may serve to prevent HIF-1 signaling. Overexpression of HIF-1α results in apoptosis (Bruick 2000; Sowter, Ratcliffe et al. 2001; Kothari, Cizeau et al. 2003). Thus, the negative feedback by HPHs during hypoxia is protective. Second, during reoxygenation, the functional importance of HPH upregulation may also be linked to hypoxic preconditioning. Hypoxic preconditioning occurs when a short duration of hypoxia is followed by reoxygenation in tissue. The heart and the brain are protected against damaging conditions of hypoxia after a period of hypoxic preconditioning (Lasley, Anderson et al. 1993; Gidday, Fitzgibbons et al. 1994; Sharp, Ran et al. 2004). While the induction of HIF-1 target survival genes are important in mediating some of the protection during preconditioning (Bergeron, Gidday et al. 2000), rapid hydroxylation of unidentified ODD-domain containing proteins during reoxygenation may also serve to prevent cell damage.

Since these enzymes catalyze an irreversible reaction, the level of enzyme expression is directly proportional to the level of enzymatic activity.

HPH-3 and FIH-1 are not Induced by Hypoxia or Hypoxia "Mimetics"

In contrast to HPH-1 and HPH-2 regulation, HPH-3 and FIH-1 were not found to be regulated by hypoxia or any other treatment that induced HPH-1 or HPH-2. We examined HPH-3 and FIH-1 regulation in human gliomas cells by qPCR analysis (Paper 1, Figure 3a) and in rat brain tissue by in situ hybridization (Paper 3, Figure 6, FIH-1 data not shown). However, HPH-3 mRNA expression levels are different in rat primary cortical astrocytes compared to neurons and in rat tissues between various brain regions (Paper 3, Figure 6 and 8). FIH-1 expression is also different between various brain regions (data not shown). Despite the absence of HPH-3 and FIH-1 regulation by HIF-1, these results implicate regulation is possible and these genes are not constitutively expressed. A recent study implicates estrogen as a regulator of HPH-3 expression (Appelhoff, Tian et al. 2004), and thus studies in nuclear hormone signaling HPH gene regulation may be important in understanding expression differences of HPH-3 in specific cells and tissues.

2-oxoacid Inactivation of HPH homologues Activate HIF-1α

Our laboratory previously described that HIF-1α stability, HIF-1 nuclear translocation and HIF-mediated gene expression in human glioma cell lines can be stimulated by

pyruvate independently of hypoxia (Lu, Forbes et al. 2002). We recently discovered that other 2-oxoacids can also activate HIF-1 independently of hypoxia in various human and rat permanent cells lines and normal cells (Paper 2, Figure 1). We used a live cell assay for oxygen-dependent HIF-1α degradation to investigate the site of action for 2-oxoacid mediated HIF-1α activiation. C6 rat glioma cells with stable ODD-GFP protein is hydroxylated and degraded in an oxygen dependent manner similar to HIF-1α. Hypoxia-mediated ODD-GFP accumulation is also stimulated by glucose, and the 2-oxoacids pyruvate, and oxaloacetate, but not by succinate (Paper 2, Figure 3c). We also found that ascorbate could reverse the activation of HIF-1 accomplished by 2-oxoacids (Paper 2, Figure 1d). Ascorbate has been shown to specifically reverse non-hypoxia mediated HIF-1α activiation (Knowles, Raval et al. 2003) and increases HPH enzymatic hydroxylation of HIF-1α proline residues (Dalgard, Lu et al. 2004; Salnikow, Donald et al. 2004). These results suggested that endogenous 2-oxoacids blocked oxygen-dependent degradation at the site of HPH enzyme action.

We proceeded to study the mechanism of action by which 2-oxoacids HPH hydroxylation of HIF-1α. We determined that pyruvate and oxaloacetate interact with the HPH homologues by utilizing a binding assay with immobilized 2-oxoglutarate and *in vitro* translated radiolabelled HPH proteins. Pyruvate and oxaloacetate displaced the iron dependent binding of radiolabelled-HPHs to immobilized 2-OG (Paper 2, Figure 4d), suggesting that these 2-oxoacids can interact with the HPH enzyme active site. Most importantly, we detected inhibition of HPH enzymatic activity by pyruvate and oxaloacetate for all three homologues (Paper 2, Figure 4h and S1). Interestingly, 2-oxoacid inhibition of HPH enzymatic activity was influenced by the concentration of

ascorbate present in the assay (Paper 2, Figure 4H and S1). Though other studies have demonstrated non-hypoxia HIF induction by growth factor (Semenza 2003), reactive oxygen species (Gerald, Berra et al. 2004), and nitric oxide signaling (Metzen, Zhou et al. 2003), we have identified an additional manner by which HIF-1α can accumulate in normoxic conditions, i.e. through signaling by cellular metabolites. In fact, a recent study implicates succinate, a TCA cycle metabolite, as a HIF-1 activator through inhibition of the HPHs (Selak, Armour et al. 2005). However, we did not find succinate to activiate HIF-1 in any of our investigations. In many cancers, aerobic glycolysis occurs despite normal oxygen availability (Garber 2004). In these settings, a high concentration of glycolytic metabolites may cause a constant activation of HIF-1, which in turn may promote additional aerobic glycolysis through upregulation of glucose transporters and glycolytic enzymes. This feed-forward signaling system may serve as the basis for high basal HIF-1α levels found in some cancers and the high malignant profile of these tumors.

HPH Brain Expression Patterns Are Differential and Overlapping

The first characterization of HPH homologues in mammalian tissues showed differential and overlapping expression (Lieb, Menzies et al. 2002). Our results characterizing HPH expression in the rat also yielded differential and overlapping expression among various brain regions. Our finding that all HPH homologues are high or moderately expressed in the cerebellum helps explain the high HPH hydroxylation capacity found in that brain region compared to the others investigated. In the same manner, we found that HPH homologue expression was lowest in the cerebral cortex, in

correlation with low HPH hydroxylation capacity. Additionally, the expression distribution of individual HPH homologues in the brain may provide insight into HPH expression in specific brain cell nuclei and populations. For example, HPH-2 was the only homologue found to be highly expressed in pontine nuclei. HPH-1 and HPH-3 also demonstrated sole expression in other specific brain areas. This initial *in situ* hybridization analysis is now being followed by a more detailed analysis inspecting these areas specifically. Emulsion development of HPH in situ hybridization slides and colocalization with antibodies for cell markers will provide identification of the specific cell types that express these HPHs.

What is the Functional Significance of HPH Redundancy?

The functional significance of having multiple HIF-1 associated oxygen sensors remains unclear. The obvious evolutionary theory for having several HPH homologues is to provide for the ability to respond to the large gradient of oxygen availability that may occur in physiological conditions. Each HPH homologue could possess a different K_m for molecular oxygen, and thus could be active at different oxygen tensions. Recent findings do not support this theory, as all HPHs were found to possess a K_m of approximately 230 μM (Hirsila, Koivunen et al. 2003), thus responding to changing oxygen tensions in the same manner. Additionally, the K_m for the cosubstrate 2-oxoglutarate and the cofactor ascorbate is ~60 and ~170, respectively, for all three enzymes. However, the HPH enzymes do differ in their ability to hydroxylate the two critical proline residues of HIF-1α. The three enzymes possess different rates of enzymatic hydroxylation in the order of HPH2 = HPH1 > HPH3 (Tuckerman, Zhao et al.

2004). Also, HPH-1 only hydroxylates proline-564 (Epstein, Gleadle et al. 2001). These differences were found to be explained by the K_m of these enzymes for their target protein, which varied for each HPH homologue. Thus, the reason for having redundant HPH homologues in the mammalian genome may not lie in the basic mechanisms of catalysis of these enzymes, but rather the differential ability of each homologue to hydroxylate proline residues on specific target proteins. Currently, HIF alpha subunits are the only known target of the HPH homologues. However, evidence now exists for additional targets. The large subunit of RNA polymerase II is found to be hydroxylated at a proline residue before being targeted for ubiquitin mediated degradation by pVHL (Kuznetsova, Meller et al. 2003). The iron regulatory protein-2 is regulated by hypoxia and hypoxia "mimetics" in the same manner as HIF-1α (Hanson, Rawlins et al. 2003). Skp1, a E3 ubiquitin ligase adapter, has also been found to be hydroxylated at a proline residue in *Dictyostelium*, by a protein similar to the HPHs(van der Wel, Ercan et al. 2005). Further investigation may demonstrate that mammalian Skp1 is a target for one or more of the HPH homologues.

In summary, this thesis investigated and answered questions involving the regulation (Paper 1), biochemistry (Paper 2), and expression (Paper 3) of the HPH homologues in mammalian brain cells and tissues. It is our hope that these findings will increase scientific understanding of oxygen-sensing mechanisms and adaptations in the brain, and lead to new studies, therapies, and tools for treating oxygen-related neurological diseases such as stroke, cancer, heart failure, among others.

BIBLIOGRAPHY

Appelhoff, R. J., Y. M. Tian, et al. (2004). "Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor." J Biol Chem **279**(37): 38458-65.

Bergeron, M., J. M. Gidday, et al. (2000). "Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain." Ann Neurol **48**(3): 285-96.

Bernaudin, M., Y. Tang, et al. (2002). "Brain genomic response following hypoxia and re-oxygenation in the neonatal rat. Identification of genes that might contribute to hypoxia-induced ischemic tolerance." J Biol Chem **277**(42): 39728-38.

Bickler, P. E. and P. H. Donohoe (2002). "Adaptive responses of vertebrate neurons to hypoxia." J Exp Biol **205**(Pt 23): 3579-86.

Bruick, R. K. (2000). "Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia." Proc Natl Acad Sci U S A **97**(16): 9082-7.

Bruick, R. K. and S. L. McKnight (2001). "A conserved family of prolyl-4-hydroxylases that modify HIF." Science **294**(5545): 1337-40.

Carrero, P., K. Okamoto, et al. (2000). "Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha." Mol Cell Biol **20**(1): 402-15.

Czyzyk-Krzeska, M. F. (1997). "Molecular aspects of oxygen sensing in physiological adaptation to hypoxia." Respir Physiol **110**(2-3): 99-111.

Dalgard, C. L., H. Lu, et al. (2004). "Endogenous 2-oxoacids differentially regulate expression of oxygen sensors." Biochem J **380**(Pt 2): 419-24.

Dijkhuizen, R. M., S. Knollema, et al. (1998). "Dynamics of cerebral tissue injury and perfusion after temporary hypoxia-ischemia in the rat: evidence for region-specific sensitivity and delayed damage." Stroke **29**(3): 695-704.

Donohoe, P. H., C. S. Fahlman, et al. (2001). "Neuroprotection and intracellular Ca2+ modulation with fructose-1,6-bisphosphate during in vitro hypoxia-ischemia involves phospholipase C-dependent signaling." Brain Res **917**(2): 158-66.

Epstein, A. C., J. M. Gleadle, et al. (2001). "C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation." Cell **107**(1): 43-54.

Fearon, I. M., A. C. Palmer, et al. (1999). "Modulation of recombinant human cardiac L-type Ca2+ channel alpha1C subunits by redox agents and hypoxia." J Physiol **514 (Pt 3)**: 629-37.

Fearon, I. M., A. C. Palmer, et al. (2000). "Hypoxic and redox inhibition of the human cardiac L-type Ca2+ channel." Adv Exp Med Biol **475**: 209-18.

Fearon, I. M., A. D. Randall, et al. (2000). "Modulation of recombinant T-type Ca2+ channels by hypoxia and glutathione." Pflugers Arch **441**(2-3): 181-8.

Garber, K. (2004). "Energy boost: the Warburg effect returns in a new theory of cancer." J Natl Cancer Inst **96**(24): 1805-6.

Gerald, D., E. Berra, et al. (2004). "JunD reduces tumor angiogenesis by protecting cells from oxidative stress." Cell **118**(6): 781-94.

Gidday, J. M., J. C. Fitzgibbons, et al. (1994). "Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat." Neurosci Lett **168**(1-2): 221-4.

Haddad, J. J. and S. C. Land (2000). "O(2)-evoked regulation of HIF-1alpha and NF-kappaB in perinatal lung epithelium requires glutathione biosynthesis." Am J Physiol Lung Cell Mol Physiol **278**(3): L492-503.

Hanson, E. S., M. L. Rawlins, et al. (2003). "Oxygen and iron regulation of iron regulatory protein 2." J Biol Chem **278**(41): 40337-42.

Hirose, K., M. Morita, et al. (1996). "cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt)." Mol Cell Biol **16**(4): 1706-13.

Hirsila, M., P. Koivunen, et al. (2003). "Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor." J Biol Chem **278**(33): 30772-80.

Hochachka, P. W. and P. L. Lutz (2001). "Mechanism, origin, and evolution of anoxia tolerance in animals." Comp Biochem Physiol B Biochem Mol Biol **130**(4): 435-59.

Huang, L. E., J. Gu, et al. (1998). "Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway." Proc Natl Acad Sci U S A **95**(14): 7987-92.

Jaakkola, P., D. R. Mole, et al. (2001). "Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation." Science **292**(5516): 468-72.

Jiang, B. H., G. L. Semenza, et al. (1996). "Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension." Am J Physiol **271**(4 Pt 1): C1172-80.

Kallio, P. J., K. Okamoto, et al. (1998). "Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha." Embo J **17**(22): 6573-86.

Kawaguchi, C., S. Takizawa, et al. (2002). "Regional vulnerability to chronic hypoxia and chronic hypoperfusion in the rat brain." Pathophysiology **8**(4): 249-253.

Knowles, H. J., R. R. Raval, et al. (2003). "Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells." Cancer Res **63**(8): 1764-8.

Kothari, S., J. Cizeau, et al. (2003). "BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF." Oncogene **22**(30): 4734-44.

Krieg, M., R. Haas, et al. (2000). "Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function." Oncogene **19**(48): 5435-43.

Kung, A. L., S. Wang, et al. (2000). "Suppression of tumor growth through disruption of hypoxia-inducible transcription." Nat Med **6**(12): 1335-40.

Kuznetsova, A. V., J. Meller, et al. (2003). "von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination." Proc Natl Acad Sci U S A **100**(5): 2706-11.

Lando, D., D. J. Peet, et al. (2002). "FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor." Genes Dev **16**(12): 1466-71.

Lasley, R. D., G. M. Anderson, et al. (1993). "Ischaemic and hypoxic preconditioning enhance postischaemic recovery of function in the rat heart." Cardiovasc Res **27**(4): 565-70.

Lewis, A., M. E. Hartness, et al. (2001). "Recombinant hTASK1 is an O(2)-sensitive K(+) channel." Biochem Biophys Res Commun **285**(5): 1290-4.

Lieb, M. E., K. Menzies, et al. (2002). "Mammalian EGLN genes have distinct patterns of mRNA expression and regulation." Biochem Cell Biol **80**(4): 421-6.

Lu, H., R. A. Forbes, et al. (2002). "Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis." J Biol Chem **277**(26): 23111-5.

Mahon, P. C., K. Hirota, et al. (2001). "FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity." Genes Dev **15**(20): 2675-86.

Marrif, H. and B. H. Juurlink (1999). "Astrocytes respond to hypoxia by increasing glycolytic capacity." J Neurosci Res **57**(2): 255-60.

Metzen, E., U. Berchner-Pfannschmidt, et al. (2003). "Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing." J Cell Sci **116**(Pt 7): 1319-26.

Metzen, E., J. Zhou, et al. (2003). "Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases." Mol Biol Cell **14**(8): 3470-81.

Ouiddir, A., C. Planes, et al. (1999). "Hypoxia upregulates activity and expression of the glucose transporter GLUT1 in alveolar epithelial cells." Am J Respir Cell Mol Biol **21**(6): 710-8.

Pugh, C. W., J. F. O'Rourke, et al. (1997). "Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit." J Biol Chem **272**(17): 11205-14.

Ruscher, K., D. Freyer, et al. (2002). "Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model." J Neurosci **22**(23): 10291-301.

Salceda, S. and J. Caro (1997). "Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes." J Biol Chem **272**(36): 22642-7.

Salnikow, K., S. P. Donald, et al. (2004). "Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress." J Biol Chem **279**(39): 40337-44.

Sang, N., J. Fang, et al. (2002). "Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent,

hydroxylation-regulated association with p300/CBP." Mol Cell Biol **22**(9): 2984-92.

Selak, M. A., S. M. Armour, et al. (2005). "Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase." Cancer Cell **7**(1): 77-85.

Semenza, G. L. (1999). "Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1." Annu Rev Cell Dev Biol **15**: 551-78.

Semenza, G. L. (2003). "Targeting HIF-1 for cancer therapy." Nat Rev Cancer **3**(10): 721-32.

Semenza, G. L., P. H. Roth, et al. (1994). "Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1." J Biol Chem **269**(38): 23757-63.

Sharp, F. R., R. Ran, et al. (2004). "Hypoxic Preconditioning Protects against Ischemic Brain Injury." Neurorx **1**(1): 26-35.

Sowter, H. M., P. J. Ratcliffe, et al. (2001). "HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors." Cancer Res **61**(18): 6669-73.

Tam, T. F., R. Leung-Toung, et al. (2003). "Iron chelator research: past, present, and future." Curr Med Chem **10**(12): 983-95.

Tuckerman, J. R., Y. Zhao, et al. (2004). "Determination and comparison of specific activity of the HIF-prolyl hydroxylases." FEBS Lett **576**(1-2): 145-50.

van der Wel, H., A. Ercan, et al. (2005). "The Skp1 prolyl hydroxylase of dictyostelium is related to the HIFa-class of animal prolyl 4-hydroxylases." J Biol Chem.

Wang, G. L., B. H. Jiang, et al. (1995). "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension." Proc Natl Acad Sci U S A **92**(12): 5510-4.

Wang, G. L. and G. L. Semenza (1993). "Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction." Blood **82**(12): 3610-5.

Wang, G. L. and G. L. Semenza (1993). "General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia." Proc Natl Acad Sci U S A **90**(9): 4304-8.

Wenger, R. H. and M. Gassmann (1997). "Oxygen(es) and the hypoxia-inducible factor-1." Biol Chem **378**(7): 609-16.

Wenger, R. H., I. Kvietikova, et al. (1997). "Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level." Kidney Int **51**(2): 560-3.

Wick, A., W. Wick, et al. (2002). "Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt." J Neurosci **22**(15): 6401-7.